UNIVERSITY OF LIVERPOOL

Gut feeling: Role of the microbiome and immune response in *Campylobacter* colonisation of the chicken

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This thesis is based on research carried out in the Department of Infection Biology, Institute of Infection and Global Health, University of Liverpool. Except for where indicated, this thesis is my own unaided work.

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ABBREVIATIONS

AG®	Aviguard®
AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
ASV	Amplicon Sequence Variant
AvBD	Avian Beta Defensin
BCR	B cell Receptor
BSA	Bovine Serum Albumin
CAB	Colombia Blood Agar
CCDA	Charcoal-cefoperazone-deoxycholate agar
CDI	Clostridium difficile infection
CE	Competitive Exclusion
CFU	Colony Forming Unit
CMT	Caecal Microbiota Transplant
CT	Caecal Tonsil
DALY	Disability Adjusted Life Year
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EU	European Union
FAO	Food and Agriculture Organisation
FCR	Food Conversion Ratio
FMT	Faecal Microbiota Transplant
GALT	Gut Associated Lymphoid Tissue
GBS	Guillain–Barré syndrome
GI	Gastrointestinal
GIT	Gastrointestinal Tract
GPA	Gentamicin Protection Assay
HCl	Hydrochloric Acid
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IFN-γ	Interferon-γ
Ig	Immunoglobulin
iNOS	Inducible Nitric Oxide Synthase
IQR	Inter-quartile range
LB	Luria-Bertani
LDA	Linear discriminant Analysis
LOS	lipooligosaccharides
LPS	Lipopolysaccharide
MAB	Maternally derived Antibodies
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry
MALT	Mucosal Associated Lymphoid Tissue
mCCDA	Modified charcoal-cefoperazone-deoxycholate agar
M Cells	Memory Cells
MHB	Mueller Hinton Broth
MUC	Mucin

NaOH	Sodium Hydroxide
NGS	Next Generation Sequencing
NO	Nitric Oxide
OD	Optical Density
OTU	Operational Taxonomic Unit
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCoA	Principle Coordinates Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational multivariate analysis of variance
PP	Peyers Patches
PRR	Pathogen Recognition Receptors
RPM	Rotations per minute
RT-qPCR	Quantitative reverse transcription PCR
SCFA	Short Chain Fatty Acid
SPF	Specific Pathogen Free
TAE	Tris-acetate-EDTA
TGFβ	Transforming Growth Factor - β
Th	T helper Cell
TJ	Tight Junction
TLR	Toll Like Receptor
TNF-α	Tumor necrosis factor - α
UK	United Kingdom
UniFrac	Unique Fraction
US	United States
WHO	World Health Organisation

ABSTRACT

Rachel Gilroy

Campylobacter jejuni is the most frequent cause of bacterial foodborne gastroenteritis worldwide and is thought to affect ~ 600, 000 people in the UK each year alone. The preparation and consumption of poultry meat remains the single source of human *Campylobacter* infection. With over 60 % of UK retail chicken carcasses showing *Campylobacter* contamination, the poultry sector represents a crucial reservoir for human disease. Having been previously considered a commensal within avian species, infection biology of *C. jejuni* within the broiler chicken shows limited understanding. Despite numerous efforts to develop both on-farm and post-slaughter controls, these have all proven to be of limited efficacy. Therefore, an improved understanding of the infection biology of *Campylobacter* in the chicken and effective control methods are a priority

Here we used *in vivo* experimental methods to develop our understanding of the complex infection dynamics and host-microbe interactions associated with prolonged *Campylobacter* infection within a commonly used broiler chicken breed. Sampled between 2 & 28 days post-infection (d.p.i), bacteriological analysis revealed rapid *C. jejuni* colonisation of the chicken gastrointestinal tract, persisting at a high burden within the caecal crypts once established. While infrequent, evidence of systemic spread of *C. jejuni* to liver and splenic tissues was observed in all experimental trials, further confirming the invasive ability within the chicken. Early *C. jejuni* colonisation was associated with early upregulation of pro-inflammatory and Th-17 immune mediators (IL-1β, IL-6, IL17A and CXCLi2) ($p < 0.05$) caecal and caecal tonsil tissue. Prolonged *C. jejuni* colonisation from 7 d.p.i onward was instead associated with regulatory immune mediators, IL-10 and $TGF\beta_4$ ($p < 0.05$).

Modulation of the intestinal microbiome has been proposed as a potential control strategy for foodborne bacterial pathogens within poultry production, particularly as commercial chickens are reared in hatcheries with no maternal contact to develop an early or pioneer microbiome. We examined whether the at-hatch delivery of adult chicken caecal microflora (CMT) would lead to a more natural 'avian' microbiota which, in-turn, could drive an improvement in chicken gut health and reduce susceptibility to *C. jejuni* infection. Delivery of 0.1 - 0.2 ml CMT preparation (derived from 7-week old broiler chickens) within 4 hours post-hatch subsequently resulted in reduced within-flock transmission of *C. jejuni* and a reduced caecal *C. jejuni* burden ($p < 0.05$) following experimental infection compared to control birds. This response was consistently reproducible and sustained until commercial slaughter age. Compared to a commercial competitive exclusion microflora preparation (Aviguard®), CMT administration was significantly more protective against *C. jejuni* colonisation of the caeca ($p < 0.05$). 16S rRNA Illumina MiSEQ analysis showed caecal content of birds treated with CMT had higher relative abundance of *Firmicutes* taxa – namely *Ruminococcaceae* ($p < 0.05$) compared to untreated control and Aviguard® treated birds. Caecal content of CMT treated chicks showed higher community richness compared to caecal content of both control ($p < 0.001$) and Aviguard® treated chicks ($p < 0.001$). These findings indicate that a novel, at-hatch transplantation of an adult chicken microbiota might prematurely drive successional development of the chick microbiota and reduce chicken susceptibility to experimental *C. jejuni* infection more effectively than a commercial competitive exclusion product.

Chapter One: General Introduction

EVOLUTION OF THE MODERN BROILER CHICKEN

It is thought that domestication of the modern chicken (*Gallus gallus domesticus*) originated in Southeast Asia nearly 10,000 years ago (Sawai et al., 2010). While this is undisputed, a defined ancestry remains under discussion (Stevens, 1991). Charles Darwin is said to have attributed the evolution of the chicken to a single origin - that of the Red Jungle Fowl (*Gallus gallus)*(Sawai et al., 2010). While this conjecture was supported within an array of subsequent research (Delany, 2004; Fumihito et al., 1994; Lapennas & Reeves, 1983; Lawal et al., 2018), debate continued as to if this was in-fact an accurate evolutionary portrayal (Elferink et al., 2012), with many stating genetic contributions from other wild jungle fowl breeds including *Gallus varius* (green jungle fowl), *Gallus sonnerati* (grey jungle fowl) and *gallus lafayettei* (Ceylon jungle fowl) (Haas et al., 2011). Irrespective of the precise phylogenetic history that defined the modern chicken, it was their fundamental adaptability to the range of global climates that cemented their dominance over many other domesticated livestock species (Siegel, 2014). With no natural migratory behaviors and a relatively small environmental range, the extensive global geographical distribution of the domesticated chicken is intrinsically linked to human mediated dispersal (Storey et al., 2012).

Showing little reflection of current trends in poultry management, it is unlikely that the first domesticated chickens were domesticated for their ability to produce eggs or meat (Nicol, 2015). Early ancestors of todays' species would likely produce only five – six eggs within a single breeding season (Nicol, 2015). Interestingly, Liu et al. (2006) shows how distribution of chickens from a specific branch on the evolutionary tree was emulated by the distribution of the practice of cockfighting, long-outdated in many cultures, although still persisting in a number of regions including the Philippines and Thailand. It Is thought that this role in entertainment alongside religious practice provided a continued drive for domestication and distribution of fowl worldwide until mid-1800 (Al-Nasser et al., 2007; Miao et al., 2013). The transition toward the use of poultry for ornamental entertainment was likely a distinct evolutionary time-point of phylogenetic divergence (Megens & Groenen, 2012). Targeted breeding to intensify desired characteristics created specific morphological, physiological and behavioral phenotypes allowing for the creation of some 500 economical and fancy chicken breeds available today (Megens & Groenen, 2012). While some chicken breeds continued to show particular favor within poultry exhibition, others developed carcass and egg laying characteristics that would lend themselves toward intensive farming that would provide a stable source of protein for global human populations (Miao et al., 2013). Since

commercialization of chickens for these specialized production characteristics first emerged in the 19th century, this handful of breeding lines dominate almost all of today's commercial chicken population. It is thought that some of the earliest commercialized chicken breeds used in agriculture, such as White and Brown Leghorn and Rhode Island Red, were those that we today associate with intensive egg production practices (Griffin & Goddard, 1994). Continued poultry breeding to improve egg production generated an unwanted population of male chickens, as these could not be differentiated according to sex until approximately seven weeks of age (Griffin & Goddard, 1994). As such, the generation of the broiler meat industry was first demonstrated as a by-product of the egg-laying industry (Griffin & Goddard, 1994). Introduction of vent sexing technologies proved to be a point of divergence whereby breeding of chicken for meat was no longer constrained by the egg-laying industries (Griffin & Goddard, 1994). With the emergence of this distinct arm of poultry production, selection for desirable meat-producing traits gave rise to specialized broiler chicken breeds often used today. Broiler breeds began to emanate across Europe (EU) and the United States (US), largely due to their remarkably fast muscle growth and hence, ability to increase body weight within relatively short periods of time (Ganabadi et al., 2009). Today, over 100 million tonnes of poultry meat is produced each year, with comparably few specific breeds accounting for almost all of this production (Mottet & Tempio, 2017).

Initial breeding selected for traits such as growth rate, feed conversion efficiency and muscle depth relatively crudely based on observable characteristics, and so breeding pairs were often simply the largest male and female individuals available (Elfick, 2006). Although conceptually simple, this process, called mass phenotypic selection, created a pure line of selected animals and allowed for rapid generation of fast-growing broiler breeds over relatively few generations (Elfick, 2006; Wolc et al., 2015). Over approximately 60 years, the growth rate of the commercial broiler chicken has increased by over 400 % (Zuidhof et al., 2014), with a 16 week production period to market in 1950 being now reduced to just 6 – 7 weeks (Schmidt et al., 2009). This is largely thought to be a result of a 50 % improvement in the measure of efficiency of food conversion to live weight gain (Food conversion ratio) with 25 g weight gain per day in the 1950s to 100 g per day in the modern chicken (Tickle et al., 2014). It soon became clear that accompanying this continued streamlining in poultry meat production came an associated decline in reproductive performance of these individuals (Thiruvenkadan et al., 2011). The negative correlation between increase body weight of the domestic chicken and fecundity, egg hatchability and sperm cell motility was being increasingly discussed, none

of which could be compensated for by alterations in management practices (Thiruvenkadan et al. 2011). Gradually, more emphasis was placed outside the phenotypic characteristics of an individual and onto genotypic characteristics of immediate relatives known as the 'Selection Index' (Hazel, 1943; Nicol, 2015). Genetic improvement of modern commercial broilers is now largely achieved through the selection of genetic traits by only three sizeable primary broiler breeding companies – Cobb-Vantress, Aviagen and Hubbard (USDA, 2013).

THE POULTRY INDUSTRY

From the early evolution of the poultry industry, it has become the fastest growing agricultural sector worldwide. With short production cycles and food to muscle conversion efficiencies far outreaching those seen from red meat production systems, poultry benefit not only those of developed global regions, but also those limited in resource (FAO, 2013). It is in these regions that the production of poultry represents additional economic security, socio-cultural and religious importance (FAO, 2013). This being said, global production of chicken meat is dominated by China, the United States, the European Union and Brazil with this production heavily supplemented by low feed prices and rising domestic consumption (USDA, 2013). Global poultry meat consumption was recorded at 111 million tonnes in 2015, with this projected to almost 133 million tonnes by 2024 (FAO, 2013). It is thought that, of this, 89 % would be attributed solely to chicken meat to create a consumption of almost 118 million tonnes.

In the United Kingdom (UK) production of broiler chickens has continued to grow year-on - year from the first data sets publicly available in 1994 (NAW, 2018). Total poultry meat production in April 2018 was 182,800 tonnes, with broilers accounting or 90 % of total production, turkey (5%), boiling fowl (spent hens and spent breeders, 3 %) and duck (1%). A total in excess of a billion animals per annum. The value of the UK poultry industry is undeniable, with this being an industry continuing to evolve and contribute to the economy.

THE CHICKEN DIGESTIVE TRACT

It is the digestive system of any given animal that plays a vital role in conversion of feed intake into bioavailable nutrients that can be used for growth, maintenance and production processes. With the rapid growth characteristics associated with broiler breeds underlying

their importance to modern society, it is essential to understand the first point of contact between feed and broiler chicken – the avian gastrointestinal tract (GIT). While it is not possible to describe all variations between the many avian species, it is important to describe key physiological and anatomical differences between birds and mammals, and how this may contribute to various beneficial phenotypes (Denbow, 2015). The chicken digestive system is a continuous tract from the mouth (beak) to the cloaca and comprises the oesophagus, crop (extension of the oesophagus), proventriculus (glandular stomach), ventriculus or gizzard (muscular stomach), small and large intestines, caeca and rectum (Alshamy et al., 2018; Klasing, 1999; Nasrin et al., 2012). Within each intestinal anatomical section we are able to identify several sequential periods of digestion, ultimately creating a utilisable end-product that can be absorbed by the host animal (Klasing, 1999).

The oral cavity of the domestic chicken largely functions to mechanically and chemically process and lubricate ingested food, before its passage into the oesophagus. A distinct expansion of the oesophagus, commonly referred to as the crop, also exists for food storage (Klasing, 1999). The oesophagus continues from the oral cavity through to the thoracic cavity, where it terminates at the proventriculus (Madkour & Mohamed, 2019). The proventriculus, also known as the glandular stomach or 'true' stomach is the gastrointestinal site whereby digestion of the ingesta is initiated (Jacob & Pescatore, 2011). While no mechanical break-down of feed occurs here, the secretion of hydrochloric acid (HCl) and digestive enzymes from the very glandular epithelial wall primes this intgesta for entry into the gizzard (Alshamy et al., 2018). The gizzard, also known as the ventriculus or 'mechanical' stomach is a unique feature of the avian gastrointestinal tract that does not appear in mammalian anatomy (Jacob & Pescatore, 2011). With limited mechanical breakdown of feed within the mouth, the gizzard is the predominant site for such processes, with this increasing surface area available to previously added HCl and digestive enzymes (Klasing, 1999). Protecting the dense interior muscular arrangement of the gizzard from chemical digestion by these compounds is a thick protein rich secretory lining (Klasing, 1999). Specific shape and structure of the gizzard will vary between avian species according to diet, but is generally larger in size than the proventriculus within the broiler chicken (Svihus, 2014).

The avian small intestine is largely homogenous in physiological form across different species, primarily due to the considerably reduced diversity in the physical nature of ingesta following mastication by the gizzard (Klasing, 1999). It is generally accepted that the chicken small

intestinal is somewhat shorter than that of most mammalian species (Denbow, 2015). Irrespective of this disparity in size, as with mammals, the small intestine is the primary site of breakdown and absorption of carbohydrates, proteins and fatty acids (Lavin et al., 2008). The small intestine is made up of the duodenum and two further components, the jejunum and the ileum, which together form the lower small intestine (Gabriel et al., 2006). To combat the earlier addition of HCl, the pancreas secretes bicarbonate into the duodenum at point-of-entry from the ventriculus (Jacob & Pescatore, 2011). Further digestion of proteins and lipids is aided by the additional secretion of digestive enzymes from both the pancreas and gallbladder, with this creating a readily available source of simple molecules that can then be passed through to lower sections of the intestinal system (Jacob & Pescatore, 2011). These molecules are absorbed in the duodenum and jejunum whereby the intestinal mucosa is specialized with epithelial folds, or villi, to facilitate maximal absorption. Here, the afore mentioned historic selection for specific developmental trains is emphasised, with divergent anatomical intestinal features evident between traditional broiler and chicken breeds (Yamauchi & Isshiki, 1991). Through comparison of broiler and layer breed intestinal villi structure, Yamauchi & Isshiki (1991) identify tendency toward larger and more densely packed structures within broiler breeds. However, common to all breeds is the increase in absorptive capacity of over 600-fold provided by these villi and associated microvilli (Alshamy et al., 2018). A zig-zag arrangement slows intestinal flow to increase contact time with the described epithelial border, supplementing this absorptive behaviour (Pelicano et al., 2005).

Arising at the ileorectal junction are two blind-ended pouches known as the caecal crypts (Clench & Mathias, 1995). Exact functionality of the caeca remains relatively unexplored, although its importance in broiler nutrition is undeniable, with caecectomy procedures lowering food metabolisability and the digestibility of crude fibre (Denbow, 2015). It is in the caeca that water reabsorption and carbohydrate fermentation occur at maximal rate compared to other GIT regions (Svihus, 2014). A single cecum can be morphologically divided into three regions, with an epithelial layer that, although being continuous between regions, shows functional heterogeneity (Moretó & Planas, 1989). Caecal villi show greatest development at the region most proximal to the ileocecal junction, with these decreasing in size toward distal caecal regions (Svihus, 2014). Supplementary muscular structures located at ileocaecal junction are able to prevent the entrance of larger undigested molecules into the caeca, creating a faecal material of much higher water content than that of the small intestine

(Denbow, 2015). The caecal content is emptied into the large intestine for excretion up to 3 times a day (Richards et al., 2019).

The colon, occasionally referred to as the large intestine, is a section of the avian GIT linking the ileal tract with the cloacal vent whereby excreta is expelled (Denbow, 2015). Distinct to the mammalian colon, the avian colon contains a dense proportion of villi, although these are relatively flat compared to those seen in proximal intestine regions (Denbow, 2015). It is thought that the colon serves mainly in final water reabsorption as much of the utilizable nutrient source has already been derived from the faecal material by this stage (Georgaki, 2014). The colon terminates at the cloaca, which forms the final tract of the digestive (coprodeum), urinary (urodeum) and reproductive (proctodeum) systems (Georgaki, 2014) before excretion through the vent into the external environment (Georgaki, 2014).

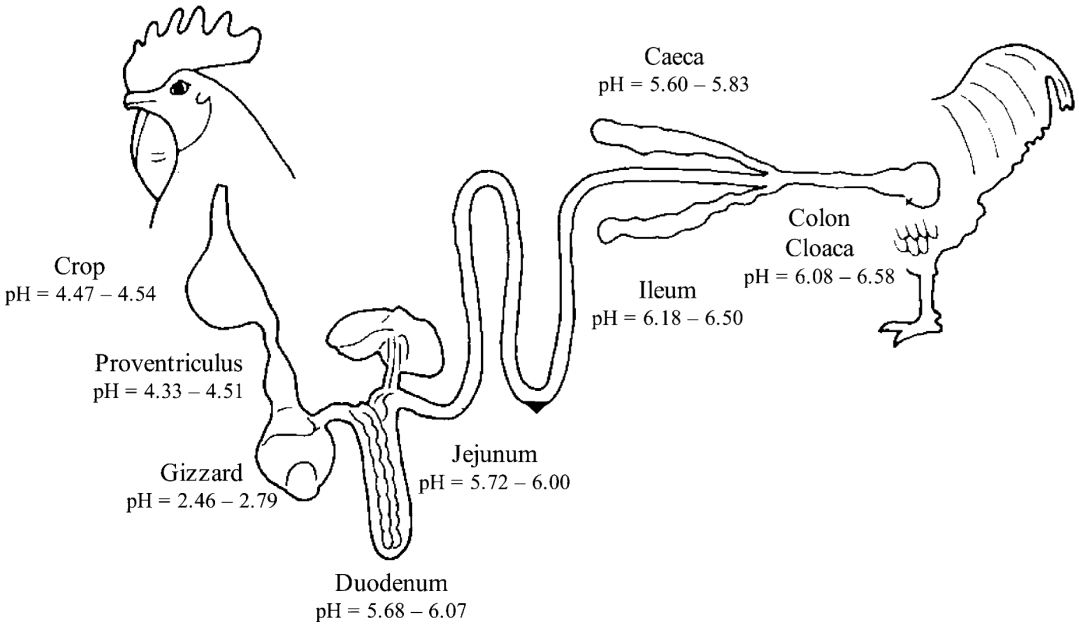


Figure 1. Diagram displaying the chicken digestive tract from crop to cloaca with associated pH values for each given region. Diagram has been attained from Gabriel et al. 2006.

THE CAECAL MICROBIAL COMMUNITY

Inhabiting the gastrointestinal tract of the chicken are microbial communities, known collectively as the intestinal microbiota (Montalto et al., 2009). This complex microbial

community is comprised of bacteria, archaea, fungi, viruses and protozoa, however with anywhere up to 10^{13} bacteria inhabiting this system, these organisms predominate (Apajalahti et al., 2008). While all sections of the intestinal tract host vast microbial communities, these communities can be differentially quantified according to anatomical region (Gabriel et al., 2006). Of these regions, the microorganisms may simply be located within the tract lumen or adhered to/embedded within the mucus layer (Albazaz & Buyukunal Bal, 2014). Of each of the GIT sites previously described, the crop and the caeca represent the primary sites of bacterial density, with 10^8 - 10^9 colony forming units per gram (CFU/g) bacteria in the crop compared to over 10^{11} within the caeca (Apajalahti et al., 2018). Accounting for all of the anatomical sections of the intestinal tract, the million-fold increases in bacterial density make the caeca an important region for microbial colonisation and as such, focus will be placed more heavily on understanding caecal microbiota as compared to other intestinal regions.

Owing to recent advances in Next Generation Sequencing (NGS), accurate microbiota characterization has facilitated more investigation into the composition of the avian caecal microbiome than ever before (Stanley et al., 2015). While early studies into the gut microbiome relied on bacteriological cultivation, we are now able to utilise an array of culture-independent techniques, including genomics, transcriptomics, proteomics and metabolomics, to provide greater information about microbial community dynamics within the intestinal tract (Arnold et al., 2018). The requirement to culture bacterial groups to allow for their identification largely constrained our understanding of community taxonomic diversity, since many bacterial species are unable to grow under such artificial conditions (Mohd Shaufi et al., 2015). With it now being understood that only 10 - 60 % of the caecal microbiota is cultivable, the introduction of more advanced molecular techniques during the early 2000s has since identified the complex chicken GIT as having over 600 bacterial species from more than 100 bacterial genera (Torok et al., 2011). While a large number of these taxonomic genera and species remain unclassified, ever-more data is emerging discerning the known taxonomic groups inhabiting the caecal flora (Torok et al., 2011).

One further facet that must be considered when performing taxonomic analysis of the broiler chicken microbiome, is the dynamic nature of this environment (Díaz-Sánchez et al., 2019). Breed, age and diet have all been identified as being strongly influential factors on the diversity and composition of the caecal microbiome (Ocejo et al., 2019). Additionally, even when continuity is maintained across all such factors, such as within commercial broiler farm

flocks, large inter-individual variation continues to exists within the bird caecal community structure (Torok et al., 2011). However, the impact of this variation appears to be largely confined to taxonomic abundance as oppose to taxonomic diversity (Ocejo et al., 2019).

It is well established that the pioneer microbiota forming within any animal occurs from parental and environmental sources immediately post - birth or hatch (Videnska et al., 2014). With a recognizable absence in parental influence upon chicks within commercial poultry rearing, the broiler chicken microbiome represents an ideal environment from which we can gain insight into the early influential behavior of specific bacterial taxons. Based on research by Ballou et al. (2016), the immature post-hatch broiler chicken caecal microbiome is characterized by low taxonomic diversity and overriding prevalence of gram negative *Enterobacteriaceae* (phylum *Proteobacteria*), with this finding commonly emulated by other research (Kubasova et al., 2019; Ocejo et al., 2019; Richards et al., 2019). Richards et al. (2019) observed taxonomic development of the caecal microbiota between 0 and 42 days post hatch (d.p.h), highlighting how this *Enterobacteriaceae* dominance may, however, be short lived, with peak relative abundance between 0 – 3 days post-hatch. The exact function of *Enterobacteriaceae* within the avian intestinal tract remains poorly determined (Grond et al., 2018). Over time, a sharp decline in *Enterobacteriaceae* exists, forming a shift toward a more diverse microbial community incorporating more gram-positive bacterial groups, namely *Clostridiales* (phylum *Firmicutes*) (Ballou et al., 2016).

By 14 d.p.h, *Ruminococcaceae* and *Lachnospiraceae* Families (phylum *Firmicutes*) comprise ~ 90 % of the caecal microbiota (Ocejo et al., 2019; Videnska et al., 2014). Both *Lachnospiraceae* and *Ruminococcaceae* have received wide attention over recent years for their favorable ability to convert complex polysaccharides within digesta to a number of short chain fatty acids (SCFAs) – particularly butyrate (Oakley et al., 2014). With heavily proposed links to maintenance of gut homeostasis and intestinal epithelial integrity alongside favorable effects on host growth, this successional shift may represent significant benefit over primary caecal taxonomic communities (Oakley et al., 2014; Ocejo et al., 2019; Vital et al., 2014). This dominant *Firmicutes* presence remains for the remaining commercial broiler life-span, however, toward the end of the production cycle (~ 42 days post hatch), a proportion of these *Firmicutes* taxons are sequentially replaced by increases in *Bacteroides* (phylum *Bacteroidetes*) (Ocejo et al., 2019; Videnska et al., 2014). While this bacterial class also shows activity in the degradation of complex polysaccharides and as such, the formation of a

subsequent energy source for avian host, this energy source is produced in the form of propionate as oppose to the previously mentioned butyrate (Videnska et al., 2014). Propionate acts as a less available source of energy and is instead linked to a more sustainable chicken growth rate against energy acquisition (Ocejo et al., 2019). At this point in development, the number of genera forming the basis of the caecal community has more than doubled compared to that of the post hatch environment, with more than 200 genera identified (Oakley et al., 2014). As such, the final picture of the caecal community is dominated by anaerobes and fewer proportions of facultative bacteria, although many of these microorganisms are yet to be classified according to taxonomic name (Lu et al., 2003).

The sequential replacement of specific taxonomic groups over time is thought to be a continual process throughout the life cycle of the commercial broiler chicken. The final picture of a caecal microbial environment dominated by *Firmicutes* with tendency toward later *Bacteroidetes* enrichment is strongly supported throughout literature, however it is undoubtable that this community structure can be influenced under the force of a number of confounding factors including breed, diet and antimicrobial use (Ocejo et al., 2019).

FUNCTION OF THE CAECAL MICROBIAL COMMUNITY

The bacterial population forming the intestinal microbiota contribute heavily to overall physiological homeostasis of the broiler chicken (Koutsos & Arias, 2006). With the immediate contact between this microbial community and the epithelial barrier of the avian host, the normal gut microbial community can have undoubted benefits, although such close contact will also be accompanied by associated physiological costs.

One a major benefit of the microbial community is one based upon competitive exclusion (CE) (Shang et al., 2018). The basis of this competitive exclusion action can form one of two arms, direct or indirect (Grond et al., 2018). Simply, the great abundance of native bacteria form strong attachment to the enterocyte epithelial wall directly limiting the availability of binding sites and nutrients for opportunistic invading pathogenic bacterial groups (Shang et al., 2018). This direct interaction of pathogenic bacterial ecology extends further through the production of antimicrobial compounds and toxins from host bacterial communities with bactericidal activities (Grond et al., 2018; Kamada et al., 2013). *In-vitro* trials have previously found the bacteriocin Reuterin produced by *Lactobacillus* species has significant impact on growth of known pathogenic species such as *Salmonella* and *Clostridium* (Yadav & Jha, 2019). As

previously discussed, specific bacterial taxa commonly inhabiting the 'healthy' chicken microbiota, generate SCFAs such as propionate, butyrate, acetate and lactate as a by-product of anaerobic metabolism (Van Der Wielen et al., 2000). The use of these SCFAs as differentially available energy substrates for host tissues is well characterized (Bedford & Gong, 2018), however the ability of these organic acids to modulate the intestinal immune system is still being uncovered. As a direct means of activity, the presence of SCFAs creates a caecal environment of lower pH, considerably less favorable for growth of many invasive pathogenic bacteria (Mani-López et al., 2012). Indirectly, SCFAs are also able to contribute to the maintenance of intestinal epithelial protection and integrity, both being key factors negatively influenced during intestinal dysbiosis, that is, a movement away from intestinal homeostasis. Specifically, SCFAs are known to induce production of the glycoprotein Mucin within the protective intestinal mucosal layer (Willemsen et al., 2003). Additionally, SCFA production is heavily associated with an increased turnover of intestinal epithelial cells (Park et al., 2016) and upregulation of tight junction assembly (Peng et al., 2009). Through antagonistic modulation of both pro- and anti- inflammatory immune responses, SCFAs are capable of generating an intestinal environment of largely tolerogenic nature (Chakravarty et al., 2019). Downregulation of potentially harmful pro-inflammatory stimuli alongside induction of anti-inflammatory cytokine Interleukin-10 (IL-10) potentially limits the immunopathologies associated with dysbiosis of the intestinal microbiota (Chakravarty et al., 2019). Thought to be associated with more direct means of interaction with the intestinal immune system, the gut microbiota has undergone further experimental exploration over recent years (Grond et al., 2018). Animals with experimentally induced germ-free intestinal tracts have been shown to have notably decreased cytokine production, systemic immunoglobulin abundance, mucus layer and relative amounts of gut-associated lymphoid tissues (GALT) (Tokuhara et al., 2019). As such, the commensal endogenous microbiota of the broiler chicken beneficially shapes the normal structure and function of the immune response to intestinal parasites (Broom & Kogut, 2018). The immediate contact of the commensal native gut flora with resident cells of the avian immune system, namely resident dendritic cells in the gut lamina propria promotes cell activation and maturation (Haghighi et al., 2005). Such activation is thought to have pronounced beneficial influence on activation of T-helper 1 (Th1) and Th2 responses, including cytokine secretion particularly associated with isotype switching of immunoglobulin classes.

In the same instance, the modulatory action of the intestinal microbial environment on the immune system comes with associated costs to the host. The vast cellular abundance of the

intestinal microbiota far exceeds that of the host animal itself and as such represents a large nutrient investment (Shang et al., 2018). Co-evolution largely maintains this nutrient competition between host and microbiota at a symbiotic nature since most host nutrient absorption occurs within the small intestine for the chicken, where bacterial density remains relatively low (Pan & Yu, 2014). However, if bacterial density within this intestinal region exceeds that of a homeostatic nature, nutrient availability tends first toward microbial species as oppose to host, creating nutrient deficit and depressed bird production efficiency (Pan & Yu, 2014; Shang et al., 2018). Extended periods of dysbiosis are thought to generate prolonged localized pro-inflammatory responses and further influence intestinal barrier function increasing the risk of pathogenic bacterial translocation across the intestinal epithelium (Shang et al., 2018).

AVIAN INTESTINAL MUCOSAL IMMUNE SYSTEM

Almost exclusively, invading pathogens will enter their host by breaching the protective surface associated with either the respiratory, reproductive or gastrointestinal tract (Kaspers et al., 2014). Since this surface area represents the largest source of contact between the host and the external environment, understanding the immunological control strategies employed as either preventative measures, or in response to pathogen breach is essential (Kaspers et al., 2014). This is of particular importance when discussing the commercial poultry industry, whereby large concentrated groups of animals exist in a localized environment, creating vulnerability of these broiler chickens for pathogenic bacteria to establish and rapidly proliferate throughout a flock. In this work we will discuss only those immune functions with relevance to pathogenic infections of the GIT.

General organization of the avian intestinal immune tissues are somewhat similar to those of mammals, in that structure will largely vary according to physiological site along the length of the tract (Yegani & Korver, 2008). The intestinal barrier itself forms the primary, physical protection from infection and is largely composed of distinct anatomical regions based upon function and cellular component (Koutsos & Arias, 2006). Forming the intestinal mucosa is the single layer of epithelial cells, itself covered by a layer of protective mucus and the underlying lamina propria, housing widespread immune components (Kato and Owen 2005). The epithelial layer and its associated basement membrane form large hairpin protrusions, commonly referred to as villi, into the lumen of the intestinal cavity increasing the exposed surface area (Smith et al ., 2014). The mechanical protection offered by this disruptive barrier

of columnar cells is strengthened by intracellular junctional complexes, the most apical being tight junctions (TJ) (Guo et al., 2018). Serving to preserve the structural integrity and regulate paracellular permeability to larger molecules within the intestinal milieu, these multi-protein complexes are crucial to effective intestinal function (Awad et al., 2017).

Nestled within this epithelial barrier are a series of specialized secretory cells, goblet cells, able to produce and secrete a mucus gel to overlay, lubricate and protect this barrier (Smirnov et al., 2005). Although thought to vary in thickness according to both intestinal site and avian species, the mucus layer is invariably predominated by a heavily modified glycoprotein, mucin (Koutsos & Arias, 2006; Zhang et al., 2015). Acting largely as a physico-chemical barrier to infection, mucin works to prevent interaction between invading microbial agent and the host epithelial surface (Cornick et al., 2015; K. Zhang et al., 2015), with this theory supported by mouse models in work from Ermund et al. (2013). It has been suggested by Koutsos and Arias (2006) that this mucosal layer may also influence the lymphoid tissues underlying this epithelial barrier.

The avian gut houses numerous types of immune cells, each differing in proportion and function; heterophils, macrophages, dendritic cells, natural killer (NK) cells, and B and T lymphocytes. Extending upon this is a group of intraepithelial lymphocytes comprising a series of Natural Killer (NK) cells and T-cells expressing either γδ or αβ form receptors (Lillehoj, 1994). T cell populations dominate such IEL communities, overshadowing both B cell and heterophil contribution (Smith & Beal, 2008). Underlying the epithelial layer described is the structural lamina propria, showing preference for B and T cell phenotypes, with these T cells largely being γδ type . γδ TCR are still subject to continuing research, however conjecture states their function in cytotoxicity and immunoregulation. The T cells inhabiting both the epithelial layers and associated lamina propria are thought to show localised polarization according to function, with CD8+ (Cytotoxic T cells) predominating within the epithelial layer and CD4+ (T helper cells) within the lamina propria (Koutsos & Arias, 2006). B cells located here secrete quantities of IgA into intestinal fluid.

Forming one arm of the diverse mucosal associated lymphoid tissues (MALTs), the GALT is a series of both diffuse and structured aggregates scattered along the entirety of the intestinal tract (Lillehoj & Trout, 1996). Of note when discussing the avian lymphoid system, is the distinct lack of structured lymph nodes compared to mammalian counterparts, only

comprising structured tonsils at some anatomical locations (Casteleyn et al., 2010; Nochi et al., 2018; Smith et al., 2014). Focusing solely on distal GIT anatomical regions, the GALTs that functionally predominate are the Peyer's patches (PP), caecal tonsils (CT), bursa of fabricius and diffuse lymphoid aggregates within the coprodeum and proctodeum (Casteleyn et al., 2010; Yegani & Korver, 2008). The B and T lymphocytes that aggregate to form these tissues have migrated at the point of functional capability from two primary lymphoid organs – Bursa of fabricius (B lymphocytes) and the Thymus (T lymphocytes) (Sklan, 2005; Smith et al., 2014). PP's are lymphoid aggregates found primarily within the lamina propria and comprise a specialized lymphoepithelial layer overlaying follicular structures (Lillehoj & Trout, 1996; Smith et al., 2014). Micro-fold (M) cells located within the lymphoepithelium are able to use apical projections to identify and present foreign antigenic material to the underlying lymphoid aggregations while also possessed a large quantity of vacuoles, reflecting their pinocytotic ability (Casteleyn et al., 2010). The trans-epithelial transport function of these cells has been highlighted in previous research, with M-cells representing a major route of entry across the gastrointestinal epithelial barrier for *Salmonella* species via trans-cellular endocytosis (Corr et al 2007). Such processes should also be considered for potential passage of *Campylobacter jejuni* from the intestinal lumen. It is within these PP sites that the primary induction of an IgA response to antigenic material is derived (Lillehoj & Trout, 1996). Thought to be largely similar in structure to the PP's, CT tissues are located at the ileocaecal junction (Befus et al., 1980). Accompanying the T and B cell populations are differentiated B cells, or plasma cells, expressing surface IgM, IgY and IgA (Lillehoj & Trout, 1996). The immune structures distributed along the intestinal tract work in accordance to induce appropriate immune-mediated responses to the continual array of challenge from ingested material and microorganisms that transition the intestinal system (Yegani & Korver, 2008). It is thought that the GALT comprises more lymphocytes than the sum of all other lymphoid tissues collectively, highlighting the risk of exposure to pathogenic challenge comparable to other anatomical sites (Smith et al., 2014).

CAMPYLOBACTER JEJUNI CHARACTERIZATION

The earliest record of the genus *Campylobacter* is thought to stem from a series of articles published by Theodor Escherich in 1886 describing the isolation of a spiral bacteria from the colons of a number of children thought to have died from 'cholera infantum' (Escherich, 1886). However, culture of this novel bacterium was unsuccessful and much of this work remained unrecognized until 1985 (Butzler, 2004). As a result, it was over 20 years later in 1909, that

attention was once again placed on this *vibrio*-link bacterium following diagnostic testing of a number of epizootic abortions in ewes by McFadyean and Stockman (Skirrow, 1994). Together with this, Jones et al., (1931) would later attribute an episode of winter dysentery in cattle to a 'Vibrio' bacterium given nomenclature *Vibrio jejuni*. The designation of *Vibrio* would later be renamed to *Campylobacter* (from the Greek and Latin meaning 'curved rod') In subsequent work published by Sebald & Veron (1963) following fermentative metabolism and DNA base composition testing distinguishing them from true *Vibrio* species (On, 2001). A further ten years later and four distinct *Campylobacter* species were described in comprehensive work by Veron & Chatelain (1973)– *C. fetus, C coli, C. jejuni and C. sputorum.*

Campylobacter belongs to the Class epsilon *Proteobacteria*, a member of the Order *Campylobacteriales* (Young et al., 2007). At present, the genus *Campylobacter* has a total of 39 names species and a further 16 subspecies (LPSN, 2019). The Genus *Campylobacter* encompasses Gram-negative, mostly microaerophilic bacterial species of small rod-like shape (0.2 - 0.8 µm x 0.5 - 5 µm) (Silva et al., 2011; Yi & Anderson, 2017). The microaerophilic nature of these bacterial species makes them ideal candidates for occupying intestinal environmental niches of limited oxygen supply (D. J. Kelly, 2001). Generally, *Campylobacter* species have fast corkscrew-like motility emanating from unsheathe flagella located at one or both ends of the bacterium (Silva et al., 2011). The *Campylobacter* species associated with human infection are thermophilic, with an optimal growth temperature of 41.5°C, although this is known to range anywhere from 30 – 46 °C (Silva et al., 2011).

Since *Campylobacter* species are not readily culturable using conventional media, various selective culture medias have been developed largely on the basis of isolating *Campylobacter jejuni* from faeces during laboratory cultivation, with these forming moist, grey colonies on such media (Corry et al., 1995). Charcoal cefoperazone deoxycholate (CCDA) agars are most often the media utilised incubated at 42°C for 48 - hours, being able to culture comparatively more *Campylobacter* strains (Silva et al., 2011). CCDA media is often further supplemented with specific levels of antibiotics (mCCDA) allowing for further selective pressures (Silva et al., 2011). Being obligate microaerophiles, *Campylobacter* species are grown under conditions off 10 % CO_2 and 5 % O_2.

Alternative diagnostic tools are often used to both rapidly identify and differentiate *Campylobacter* species. Most valuable to the food safety industry, molecular based methods such as Polymerase Chain Reaction (PCR) are able to sensitively identify *Campylobacter*

organisms using a variety of species-specific gene sequences including 16S rRNA, glyA (serine hydroxymethyltransferase) and 23S rRNA (Ricke et al., 2019; Wang et al., 2002). Immune based methods such as Enzyme Linked Immunosorbent Assay (ELISA) and Flow cytometry testing are useful when large sample groups are processed, such as in epidemiological studies, however these methods require dedication of both increased time and expense (Ricke et al., 2019) and often lack sensitivity due to cross-reactivity. More recent identification of *Campylobacter* species through matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry has yielded both rapid and accurate means of identification (Bessède et al., 2011).

ECONOMIC AND PUBLIC HEALTH IMPACT OF CAMPYLOBACTERIOSIS IN THE UK

Campylobacter remains the leading cause of human foodborne bacterial gastroenteritis worldwide, referred to as campylobacteriosis upon diagnosis (Rushton et al., 2019). campylobacteriosis is commonly characterized by severe diarrhea and abdominal cramping and is generally self-limiting in nature (Barrett et al., 2018). While human cases of *Campylobacter* rarely exceed durations of one week, however, in some cases these symptoms can develop into post-infectious sequelae presenting as Guillan-Barré syndrome, reactive arthritis, and irritable bowel syndrome (Barrett et al., 2018). Of the numerous *Campylobacter* species previously highlighted, *C. jejuni* and *C. coli* are together the most common cause of campylobacteriosis cases comprising 80 % and 10 % of total human cases respectively (BIOHAZ, 2011).

In 2017, Public Health England reported an infection rate within England and Wales of 95.57 per 100, 000 population, with this equating to 56,729 campylobacteriosis cases over that same year (PHE, 2017). Incidence at this given level represents an increase on 2016 by 4,358 cases (PHE, 2017). While this undoubtedly represents concern within the UK, of equal importance is the endemic nature of *Campylobacter* infection within those inhabiting developing countries (Barrett et al., 2018; Rushton et al., 2019). Most vulnerable to infection within these regions are infants of less than a year in age, with Rushton et al. (2019) reporting isolation rates of 8 to 21 % of all diarrhea samples.

It is undeniable that *Campylobacter* is a causative agent of widespread morbidity and mortality. A commonly used metric to assess the burden of disease is the Disability Adjusted Life Year (DALY) with this combining the effects of disease morbidity and mortality on the

health of a specific population (Mangen et al., 2018). As of 2011, the DALY impact of campylobacteriosis across the EU was recorded as € 0.35 million per year, with a total economic burden of over € 2.4 billion (European Food Safety Authority, 2014). It is further estimated that the significant under-reporting of disease associated with campylobacteriosis creating disparity in the region of 10 to 100 times, with interpolated values of over 9 million cases of campylobacteriosis in the EU per year (European Food Safety Authority, 2014). Considering the high number of true campylobacteriosis cases, the burden of human *Campylobacter* infection cannot be underestimated (European Food Safety Authority, 2014). A comprehensive understanding of campylobacteriosis prevalence is essential in predicting the impact from disease burden. A seasonal pattern of *Campylobacter* contamination has been characterized in previous work, showing strong fluctuations in disease reporting according to calendar month (Friedrich et al., 2016). In western countries, seasonal peaks of *Campylobacter* reporting are observed in July, although this can also range to August in some climates (Rushton et al., 2019; Sibanda et al., 2018). The exact reasoning behind this seasonal fluctuation in human *Campylobacter* cases are still undefined, however, some inference has been lent toward specific changes in human behavior and environmental climate alongside an increase in pathogen vector reservoirs (Sibanda et al., 2018).

SOURCES OF HUMAN CAMPYLOBACTERIOSIS

It is thought that the majority of human campylobacteriosis cases can be attributed to a food or water-borne source (Mughini Gras et al., 2012; Pitkanen & Hanninen, 2017). Of these factors, food-borne transmission tends to dominate discussion, however the is growing evidence that environmental contamination of water sources with waste from *C. jejuni* infected animals has significant impact on levels of exposure (Wagenaar et al., 2013). While unable to grow in natural environments outside of the host species, survival of viable *C. jejuni* cells has been recorded for nearly 3 months in contaminated slurries and water containing contaminated animal waste (Nicholson et al., 2005). Direct contact with such contaminated animals has also been widely identified as a transmission pathway in human infection (Wagenaar et al., 2013) and further fly-borne transmission is likely to exacerbate such infection prevalence (Pitkanen & Hanninen, 2017).

C. jejuni and *C. coli* are intestinal inhabitants within specific animal groups and have been isolated from a wide selection of warm-blooded species (Wagenaar et al., 2013). While the presence of *Campylobacter* within these species is thought to be of minor concern to

veterinary health, its carriage acts as an importance reservoir for human infection. Although this broad host range extends from wildlife to domesticated and pet animals, the particular burden found within commercial livestock animals cannot be negated. Using data collected during a national survey of livestock at slaughter in the UK (2003), either *C. jejuni* or *C. coli* were isolated from 54.6 % of cattle, 43.8 % of sheep and 69.3 % of pigs (Wagenaar et al., 2015). When assessing the capability of *Campylobacter* species to infiltrate food production systems, poultry in particular harbor vast *Campylobacter* burden (Wagenaar et al., 2015). It is thought that 50 - 80 % of all human cases of campylobacteriosis stem from the chicken *Campylobacter* reservoir, with 20 - 30 % solely emanating from preparation and consumption of broiler chicken meat (European Food Safety Authority, 2014).

CAMPYLOBACTER IN THE BROILER CHICKEN

Avian species, including commercial chicken breeds represent species with average body temperatures significantly above that of other mammalian animals (Duffy & Dykes, 2006). With stocking densities 0f 10,000 – 100,000 birds per house in commercial poultry farms across developed countries, this environment provides a perfect reservoir for optimal *Campylobacter* growth and transmission (Pitkanen & Hanninen, 2017). Over 30 years of epidemiological study has continued to portray chickens as the most important sources of human campylobacteriosis worldwide (Skirrow, 1994; Wagenaar et al., 2015). *C. jejuni* is both the most commonly isolated *Campylobacter* species in humans and chickens (Pielsticker et al., 2012), reported to make up in excess of 65 % of chicken *Campylobacter* infection (Marotta et al., 2015).

The term 'farm-to-fork' is often applied to the processes of meat production and encompasses all stages from primary production at rearing farms, transport, processing at slaughter, dressing and processing, retail and consumption of final meat products (Skarp et al., 2016). This integrated process provides multiple *Campylobacter* entry points and subsequent roles in transmission (Skarp et al., 2016). Ingestion of as little as 35 Colony Forming Units (CFU) of *Campylobacter* is sufficient to establish a colonizing population within 24 hours, with this showing rapid transmission throughout flocks (Stern et al., 1988). Once faecal shedding is established, almost all birds (> 95 %) within the flock of immediate contact will show colonisation in a matter of days (Stern, 2008). When established, colonisation tends to be sustained until the point of slaughter. While *Campylobacter* prevalence throughout the various rearing stages will vary from flock to flock, it is well known that the prevalence of

Campylobacter within positive flocks at slaughter is high, being approximately 80 % (European Food Safety Authority, 2014; Zhang et al., 2018). Further to this, cross-contamination during this post-processing period will have influence on the whole flock processed on a single line, with this increasing flock contamination prevalence (Hayama et al., 2011).

Risk factors associated with broiler chicken *C. jejuni* infection

Chicks are generally considered to be of *Campylobacter*-negative status at the point-of-hatch, with vertical transmission appearing to offer minimal contribution to *Campylobacter* spread (Camarda et al., 2000; European Food Safety Authority, 2014). Sibanda et al. (2018) indicates some ability of *C. jejuni* penetration through the surface of the egg when contaminated with faecal material, however this work requires further development to form a well-founded argument for vertical transmission from parent to offspring within the chicken. Longitudinal studies place most interest therefore, on horizontal transfer sources, largely exacerbated by the coprophagic nature of the chicken. While some horizontal risk factors appear at risk factors in many of the surveillance studies conducted into *Campylobacter* transmission within broiler chicken flocks, other secondary factors are dependent upon further variables (Table 1).

Table 1. Risk factors commonly cited amongst published literature as being important in *C. jejuni* colonisation of poultry flocks

Risk factor	Reasoning	Citation
Increasing age	- Increase in prevalence from three weeks of age. - Increased risk of exposure with age and altered susceptibility.	(Bouwknegt et al., 2004; European Food Safety Authority, 2014; McDowell et al., 2008; Sibanda et al., 2018)
Climate	- Seasonal peak in colonisation between July-September alongside increases in temperature and rainfall - Increased ventilation and water consumption in warmer months - More flies acting as mechanical vectors	(European Food Safety Authority, 2014; Hald et al., 2008; Jore et al., 2010; Rushton et al., 2019)
Thinning practices	- Contamination of personnel and equipment - Stress promotes *Campylobacter* transmission	(Hald et al., 2008; Refrégier-Petton et al., 2001)
Farm staff/visitors	- Introduction of contamination from human traffic - Lack of due care with biosecurity processes	(Agunos et al., 2014; Newell & Fearnley, 2003)
Water and feed	- *Campylobacter* is able to survive for long periods in water - Contaminated or untreated water sources increase prevalence - Use of nipple drinkers associated with increased prevalence - Backtracking from contaminated drinkers to infect whole water line	(Arsenault et al., 2007; Axelsson-olsson et al., 2005; Sibanda et al., 2018)

Insects	- Passive transfer of faeces from other houses and/or other livestock	(Craven et al., 2000; Hald et al., 2008; Shane et al., 1985)
Wild animals	- Poor vermin control	(Arsenault et al., 2007)
Other livestock	- High risk of carriage from other positive farm livestock - Farming practices utilising multiple species have higher *Campylobacter* prevalence	(Bouwknegt et al., 2004; Hansson et al., 2010; van de Giessen et al., 1998)
Environmental contamination	- Contamination of environment can persist for many weeks - Free range flocks generally higher *Campylobacter* prevalence than conventionally raised	(Refrégier-Petton et al., 2001)
Environmental carry-over	- Inadequate disinfection of poultry house between flocks - Appears to have limited impact although may account for 10 – 20 % of new flock infection	(S. Smith et al., 2016)
Antimicrobial use	- Antimicrobial agents used as therapeutic agent to control disease	(X. Chen et al., 2010)
Stocking density and flock size	- Contradictory evidence exists between flock positivity and stocking density	(Berndtson et al., 1996; Cardinale et al., 2004)
Number of bird houses	- Increased *Campylobacter* colonisation within each poultry house with increased number of total houses on site - Cross-contamination from other houses	(Arsenault et al., 2007; McDowell et al., 2008)
Bird health	Suggested association between *Campylobacter* flock positivity and bird health	(Bull et al., 2008)

LAG PHASE IN *C. JEJUNI* INFECTION OF THE CHICKEN

With little to no evidence supporting the theory of vertical *Campylobacter* transmission from parent to offspring within the chicken, it is accepted conjecture that broiler chickens are hatched under a *Campylobacter* negative status (Berndtson et al., 1996; Newell & Fearnley, 2003). Interestingly, epidemiological study suggests that this lack of detection persists until at least 10 days post-hatch under commercial production conditions, a biological characteristic distinct to *Campylobacter i*nfection within poultry species (Newell & Fearnley, 2003). The chick at point-of-hatch is protected by a relatively naive immune system with no established gastrointestinal microbiota or mucosal immune system (Hermans et al., 2014). While microbiota development begins immediately post-hatch, the first signs of immune development begin at around 4 – 7 days post-hatch (Hermans et al., 2014). The source of protection during this period is yet to be uncovered, however its continued presence throughout longitudinal and experimental study has coined this period the name 'lag-phase'. It is reported that the delay in *Campylobacter* colonisation of the commercial broiler chicken will most commonly last at least 2 weeks, at which point *Campylobacter* burden will increase until peak load at point of slaughter (6 – 7 weeks post-hatch) (Kalupahana et al., 2013; Newell & Fearnley, 2003; Sahin et al., 2003). Comprehensive research by Damjanova et al. (2011) assessed *Campylobacter* positivity of multiple animal and environmental samples at 2 and 7

days post-hatch, including air, feed, water and faeces, with all samples returning no detectable contamination. By 6 weeks post-hatch, all birds within this study had detectable *C. jejuni* infection (Damjanova et al., 2011).

While reasons underlying this so-called lag-phase remain unclear, Berndston et al. (1996) found day old chicks exposed to a more invasive *Campylobacter* strain exhibited pathological signs of diarrhea hours after artificial infection. This work infers that the lag-phase observed within poultry against *Campylobacter* infection might be associated with an inherent facet of poultry species instead of an ecological feature of the *Campylobacter* species (Newell & Fearnley, 2003). Adding to the complexity of the physiological basis behind this phenomenon is the dynamic nature of the avian intestinal niche during the early stages post-hatch (Newell & Fearnley, 2003). Maturation of the mucosal immune system, successional changes in the intestinal microbiota and changes in commercial production management processes create an array of confounding factors occurring throughout this period, with each having a yet unknown, impact on this age-related susceptibility of the chicken to infection (Newell & Fearnley, 2003).

One concept, having been widely discussed in published literature, is the contribution of *Campylobacter*-specific maternally derived antibodies (MAB) found in abundance within chicks post-hatch before decline around 14 days post-hatch (Newell & Fearnley, 2003; Orhan Sahin et al., 2003). Sahin et al. (2003) highlighted how, of newly hatched chicks from five broiler chicken flocks, high levels of *C. jejuni* specific antibodies were detected in all chicks up to 7 days post-hatch. A series of experimental data sets present strong suggestion that MAB are able to target multiple loci on the outer membrane of *C. jejuni* supplemented by roles in complement mediated killing of these bacterial pathogens (Sahin et al., 2003). Of further interest are the commensal community assembly patterns associated with the developing chicken microbiota, with this thought to form further protective barrier in reducing susceptibility to infection. (Newell, 2002) provides evidence that specific pathogen free (SPF) birds are less resistant to experimental *Campylobacter* infection compared to commercial broiler chickens removed from production systems during the early lag phase. This understanding is further supported by Newell & Fearnley (2003) highlighting how *Campylobacter*-negative phenotype showed strong association with a birds' ability to produce specific inhibitory metabolites within the caeca. Development of a viable explanation for this

'lag-phase' period within the ecology of poultry *Campylobacter* infection could provide a vital basis from which future control strategies could be developed.

CAMPYLOBACTER INFECTION IN THE CHICKEN: ACTING AS A PATHOGEN OR A COMMENSAL?

While literature exists stressing the pathogenic nature of various opportunistic taxa known to invade the poultry gastrointestinal tract (Kaiser et al., 2006; Kaiser et al., 2000), the immunogenic ability of *Campylobacter* spp. has been significantly less explored (Meade et al., 2009). As such, there exists continued contradiction within available literature as to the assignment of *Campylobacter* spp. within the chicken to either commensal or pathogenic ecologies (Wigley, 2015). *Campylobacter* is known to colonise the chicken via the fecal-oral route, establishing strong bacterial burdens within the lower GIT, particularly the caeca whereby it can colonise to levels up to 10^9 CFU/g caecal content (Sahin et al., 2015). It is thought that this colonisation can be established rapidly within the intestinal mucus, however *Campylobacter*s may also be able to invade the intestinal epithelium and be further isolated from the bloodstream (Awad et al., 2018). As such, although the intestinal tract constitutes the main colonisation site of *C. jejuni*, dissemination and colonisation to a lesser extent has been observed in the liver, spleen, deep muscle, thymus and bursa of fabricius (Awad et al., 2018; Chaloner et al., 2014; Humphrey et al., 2014), with this invasive ability also largely reliant on infecting *Campylobacter* strain (Awad et al., 2018; Chaloner et al., 2014).

Colonisation of the broiler chicken with high burdens of *C. jejuni* is often in the absence of obvious clinical disease, resulting in a long-held opinion that colonisation is commensal and largely harmless (Humphrey et al 2014; Williams et al 2016). This poor immune activation would, in theory, result in the extensive persistence of *Campylobacter* colonisation throughout the intestinal system often seen (Meade et al. 2009; Awad et al. 2018). However, with increasing investigative research beyond that of the early descriptive studies, the stimulation of a pro-inflammatory response preceding that of a tolerogenic immunomodulatory nature has been associated with *C. jejuni* infection (Humphrey et al., 2014; Li et al., 2010; Smith et al., 2005).

This being said, work by Humphrey et al. (2014) indicates higher *Campylobacter* burden as a significant risk factor for welfare indications including hock marks and pododermatitis. Both hock marks and pododermatitis largely the result of high ammonia content on wet litter, and severely intensified in faster growing broiler breeds. Compromises in the gut health are

further eluded to in the work of Awad et al. (2018) whereby interaction of luminal *C. jejuni* with the intestinal epithelium is shown to decrease jejunal villus height and crypt depth, consequentially decreasing the available surface area for nutrient absorption. This can often result in notable decrease in animal productivity (Colles et al., 2016).

INTESTINAL MUCOSAL SURFACE ASSOCIATION WITH *C. JEJUNI*

The avian mucosal epithelial surface provides a physical barrier protecting the body's interstitium from the external environment (Júnior & Júnior, 2016). Spanning the paracellular space between endothelial cells are a collection of tight junctions, with these controlling paracellular permeability. *In-vitro* studies by Lamb-Rosteski et al. (2008) were able to identify disruption in the claudin-4 protein of endothelial tight junctions following incubation with *C. jejuni*. This mechanism is thought to increase paracellular permeability, increasing likelihood of extra intestinal spread and fluid absorption (Júnior & Júnior, 2016; Lamb-Rosteski et al., 2008).

While commonly not adhering or invading the avian intestinal epithelial layer *C. jejuni* becomes largely associated with the mucus layer lining of the avian GIT (Looft et al., 2018). A core constituent of this mucosal layer are mucin proteins, with these glycoprotein units selectively influencing cellular adhesion, invasion and immune surveillance (Júnior & Júnior, 2016). Having an active anti-inflammatory effect against Gram negative bacteria, Byrne et al. (2007) found dose-dependent attenuation *C. jejuni* using chicken intestinal mucus. While it has been observed throughout many *in-vitro* studies that *C. jejuni* is able to show invasive phenotypes against avian intestinal cells *in-vitro*, it has since been shown that addition of crude chicken mucus dampened such internalization (Byrne et al., 2007). This is in contrast to the results seen following the addition of human intestinal mucus, which showed contrasting hyper-internalization of *C. jejuni* (Byrne et al. 2007). Contribution of avian mucin glycoproteins to the attenuation of *C. jejuni* invasion within the chicken may provide some explanation as to its persistence within the avian intestinal tract.

INNATE IMMUNITY TO *CAMPYLOBACTER* INFECTION IN THE CHICKEN

As previously noted, the mature mucosal lining of the gastrointestinal tract constitutes one of the most immunocompetent region of the broiler chicken combining both innate and adaptive responses (Awad et al., 2018). It is the seqeuelae of events initiated during primary innate immune responses that are key to determining the extent of subsequent adaptive outcomes

(Meade et al., 2009). Upon entry of *Campylobacter*, this innate immune response is responsible for recognizing these microbes as distinct from self, achieved through pattern recognition receptor (PRR) - dependent mechanisms of sentinel cells within the mucosal epithelia (Júnior & Júnior, 2016). Of the PRR's, it is the Toll-like receptor cells (TLRs) that are most commonly associated with *Campylobacter* infection and act to specifically recognize invading microbial components known as pathogen associated molecular patterns (PAMPs) (Otto et al., 2012). Once activated, phagocytosis of the pathogenic microbial is promoted, alongside the activation of a cell signaling cascade (Meade et al. 2009). Work by De Zoete et al. 2010 found much of the TLR recognition of *Campylobacter* is reminiscent of that seen within human *Campylobacter* infection and predominated by TLR4 (De Zoete et al., 2010; Humphrey et al., 2014; Meade et al., 2009). Of particular importance is the activation of chicken TLR21, a recognition response not identified for the mammalian functional equivalent TLR9 during human infection (De Zoete et al., 2010; Humphrey et al., 2014; Meade et al., 2009; Shaughnessy et al., 2009). While the exact role of TLR21 following its identification of *Campylobacter* is yet to be defined, we have greater insight into the function of the more commonly referenced TLR4 (Awad et al., 2018). Activated by the gram-negative bacterial lipopolysaccharide (LPS) and bacterial DNA respectively, TLR4 and TLR21 have the ability to induce activation of multi-cellular pathways and production of antimicrobial peptides (AMPs) through production of a series of pro- and anti-inflammatory effector molecules (Awad et al., 2018; Meade et al., 2009). Induction of inducible nitric oxide synthase (iNOS), and subsequently nitric oxide (NO), within chicken HD11 macrophage cell lines has been reported by He et al. (2012) resulting from chicken TL4 and TL21 activation. Chicken NO has known bactericidal activity and as such, represents an important localized innate immune response following infection (He et al., 2012). Conversely, studies by Meade et al. 2009 have linked increases in TLR21 expression to a concomitant downregulation of multiple avian β defensin (AvBD) genes. With the AvBD family known for being potent antimicrobial peptides, their downregulation in association with *C. jejuni* infection might contribute to the prolonged colonisation ecology (Meade et al. 2009).

In mammalian epithelial and primary chick kidney cells expression of the pro-inflammatory cytokines interleukin-6 (IL-6) and IL-1β alongside the chemokine ligands CXCLi1 and CXCLi2 (orthologues of the human chemokine IL-8) is increased in response to TLR4 and TLR21 activation (Friis et al., 2009; Hermans et al., 2014). Up-regulation of the chemokine ligands CXCLi1 and CXCLi2 are also widely associated with an influx of heterophils and monocytes to

sites of inflammation (Hermans et al., 2014) while IL-1β and IL-6 are common mediators of the innate immune response and further stimulate adaptive immune defenses. Although observable within the avian caecal crypts, this response is somewhat more dilute bar that of the chemoattractant chemokines CXCLi1 and CXCLi2 (Hermans et al., 2014). While debated as to its prevalence alongside *C. jejuni* infection, reports of infiltration of pro-inflammatory immune cells into the mucosal crypts may support this chemokine surge (Humphrey et al., 2014; Larson et al., 2008).

ADAPTIVE IMMUNITY TO *CAMPYLOBACTER* INFECTION IN THE CHICKEN

Description of the adaptive responses to *C. jejuni* within the broiler chicken are limited within current research (Lacharme-Lora et al., 2017). Although sequential in timing, an adaptive response is also generated within the avian *C. jejuni* infection and is largely integrated with that of the innate response (Williams et al., 2016). Antigen presenting cells (APCs) within the mucosal epithelia are essential in recognizing and engulfing and presenting pathogen epitopes to naïve immune cells via protein structures known as major histocompatibility complex molecules (MHC) (Júnior & Júnior, 2016).

The adaptive immune response consists of an antibody mediated and a cell mediated response (Erf, 2004). Cell mediated responses are associated with adaptive T cell mechanisms, with these being divided into two further T cell subdivisions known as T helper (Th) cells (CD4+) and T cytotoxic cells (CD8+) (Júnior & Júnior, 2016). Recognition of *C. jejuni* antigens by T cells is MHC-restricted, requiring antigen presentation and cytokine production (IL-6, IL-12 and TNF-alpha) from APC's to induce subsequent Th cell polarization (Júnior & Júnior 2016). This polarization will drive either a pro-inflammatory response profile (Th1) or an anti-inflammatory response profile (Th2). More recently, a further Th17 pro-inflammatory response has been characterized. Upregulation of the Th1 profile correlated with increases in IFN-γ secretion from these cells while Th2 upregulation correlates with increases in Tumor Growth Factor- β (TGF-β), IL-4 and IL-10 secretion (Júnior & Júnior 2016). Th1 and Th17 guided responses are largely associated with attempted *C. jejuni* clearance while Th2 responses induce convalescence (Júnior & Júnior 2016). The use of mathematical modelling approached couple with cytokine expression data show the relative importance of Th17 responses across broiler breeds with both high and low inflammatory responses (Reid et al. 2017). As Th17 responses also play a key sentinel role in the gut maintaining tight junction integrity and

increasing mucin and antimicrobial peptide production, it may be these responses are key to largely restricting infection to the gut.

Humoral immune response to *C. jejuni* is reliant on B cell driven mechanisms, with these cells able to directly recognize soluble *C. jejuni* antigens by the B cell Receptor (BCR) without MHC mediated pathways (Júnior & Júnior 2016). Upon activation, B cells differentiate to form plasma cells that secrete BCR molecules more commonly referred to as antibodies, with these entering systemic circulation, mucosal surfaces and egg yolk (Júnior & Júnior 2016). Three immunoglobulin classes are known to exist, with these being avian orthologues of IgA, IgM and IgY (Júnior & Júnior 2016). IgM is largely homologous with that of the immunoglobulin group in mammals and is the first of the immunoglobulin classes produced during the humoral response (Janeway Jr, 2001). This early production is prior to cellular adaptation to the invading antigen and so is largely unspecific and of low affinity (Janeway Jr, 2001). However, IgM molecular are formed with pentomeric structure of 10 antigen-binding sites and as such, are effective activators of complement (Janeway Jr, 2001). This large pentomeric structure limits IgM localization largely to the blood (Wang et al., 2006). IgY largely works to opsonize antigenic material for immune cell phagocytosis, IgA works instead as a direct neutralizing antibody and is often the predominant immunoglobulin in secretions lining the mucosal tracts (Lamm, 1997). Of smaller size are the IgY and IgA isotypes, with these being of monomeric form (Janeway Jr, 2001). Intraepithelial lymphocytes (IELs) of the caecum, the primary site of *C. jejuni* colonisation within the chicken, have been assessed for thei potential function in local immune responses, however negligible changes in CD4+ and CD8α+ T cells have been observed (Pielsticker et al 2012). Later implication of breed effects on IEL response to *Campylobacter* in the chicken was implied by Han et al (2016) and may underly such findings. The same research observed significant effects of *C. jejuni* on LPL populations within the chicken caecum (Han et al., 2016)The exact nature of the immunoglobulin response to *Campylobacter* within the broiler chicken remains largely ill-defined and undeveloped (Wigley & Humphrey, 2014). Forming the basis of a significant portion of our knowledge is the evidentiary reaction between avian immunoglobulin classes and a number of *C. jejuni* outer membrane proteins (de Zoete et al., 2007). While such proteins are thought to include LPS and Lipooligosaccharides (LOS), the most notable remains *C. jejuni* flagellin (Sahin et al., 2001). While it is common for literature to correlate early chick MAB presence with the lag-phase commonly seen in *Campylobacter* infection of the chicken, functional studies into the exact contribution to this are scarce (Wigley & Humphrey, 2014). While continual fluctuations of all

avian immunoglobulin classes exist throughout the short commercial broiler life-cycle, it is generally accepted that *Campylobacter* colonisation of the chicken will induce an anti-*Campylobacter* immunoglobulin response (Shoaf-Sweeney et al., 2008). However, this B lymphocyte mediated response may limit the overall extent of *Campylobacter* burden but shows little evidence of complete clearance of infection within the GIT, particularly the caeca (Sahin et al., 2003; Shoaf-Sweeney et al., 2008). The incline in immunoglobulin (namely IgY) production following *Campylobacter* challenge, as seen in the work of Myszewski & Stern, (1990) may serve mainly in clearance of infection of more proximal GIT sections, such as the ileum and jejunum, as oppose to clearance of the caecal crypts (Lacharme-Lora et al., 2017). Lacharme-Lora et al. (2017) suggests that, while not inhibiting initial passage of *C. jejuni* through these sections to the caeca, immunoglobulin mediated protection might instead protect against re-colonisation of the ileum and jejunum from more distal GIT sections. Targeted influence of these immunoglobulins on caecal *C. jejuni* colonisation is instead thought to occur at much later point in broiler development, usually outside the age remit seen for broiler chickens within the commercial poultry sector (Lacharme-Lora et al., 2017).

CAMPYLOBACTER JEJUNI CONTROL METHODS

Based on published literature reviews, Lin (2009) proposed three approaches to control of *Campylobacter* within the poultry industry, with these acting as distinct therapies in their own right, but also, strategies that can be amalgamated accordingly (Lin, 2009; Wagenaar et al., 2006). In essence, it is thought that Campylobacter can be controlled through; reduction in initial exposure, enhancement of poultry protection against colonisation post-exposure and therapeutic reduction in burden following colonisation (Lin, 2009), with a number of commonly referenced strategies described in Table 2.

Table 2. Commonly referenced on-farm strategies for the control of *C. jejuni* within commercial broiler chicken farming.

Factor	*Reasoning*	*Potential limitations*	*Reference*
Biosecurity measures	General on-farm biosecurity measures including those regarding worker hygiene and physical farm barriers are required to prevent initial flock colonisation. While no one route, vehicle or vector has been identified in reducing *Campylobacter* colonisation, high biosecurity strategies have been correlated with *Campylobacter* absence.	Sustaining such stringent biosecurity measures on large, established poultry farms may not be sustainable and would require complete cooperation from all farm workers.	(Agunos et al., 2014; Lin, 2009; Meunier et al., 2016; Sibanda et al., 2018)
Competitive exclusion	Competitive exclusion therapies comprise defined or undefined preparations of commensal bacterial taxa. While the mechanisms are unclear, promising results have been observed in reducing poultry *Salmonella* infection, with contradictory results observed for *Campylobacter*.	For commercial use within the poultry industry, more needs to be understood regarding mechanistic action, alongside full characterisation of the individual species present within the complex population.	(Lin, 2009; Young et al., 2007)
Vaccination	Vaccination strategies are were largely based of theory that initial lag phase protection from *Campylobacter* colonisation within the chicken was the result of maternally derived antibody presence. There has also been some reported correlation between reduced colonisation of *C. jejuni* with high antibody titres.	Vaccination strategies are reliant on an increased understanding of avian immunological response to infection. The short production cycle used within the poultry industry limits potential efficacy of vaccine strategies employing humoral response strategies.	(de Zoete et al., 2007)
Genetic selection	Differential colonisation of chickens has been observed between chicken breeds	Individual variation in colonisation resistance is observed within-breed	(Li et al., 2008; Stern et al., 1990)
Bacteriophage	Bacteriophages (phages) are intracellular parasites able to target and lyse specific bacterial pathogens. *Campylobacter* models using oral *Campylobacter* infection of broiler chickens shows reduction in pathogen load ranging from 0 - 5 log units.	Reproducibility of beneficial impact on infection is poor, with some links being made to the development of target bacterial phage resistance. Widespread discontent amongst producers and consumers as to the phage	(Carrillo et al., 2005; Clavijo & Flórez, 2018; Connerton et al., 2018; Wernicki et al., 2017)

		therapy safety has hampered its regulation and approval.	
Bacteriocin	Bacteriocins are small molecular peptides produced by gram- positive and negative microorganisms. Several studies have reported a number of commensal bacterial species within the chicken that are able to produce bacteriocins against *Campylobacter*, with oral administration showed to reduce colonisation by 5 - 8 log units.	Fear of resistance development	(Johnson et al., 2017; Svetoch & Stern, 2010)
Prebiotics and Probiotics	These are live commensal microbial preparations and non-digestible food ingredients that promote beneficial bacterial growth within the gut. Probiotics, or probiotic/prebiotic combinations have shown promising results at preventing *Campylobacter* colonisation during primary production.	Large discrepancy in trial outcomes based on variation in methodologies. Alterations in intestinal microflora communities following therapeutic administration appear to be relatively transient.	(Kizerwetter-swida & Binek, 2005; Stanley et al., 2014)

COMPETITIVE EXCLUSION PRODUCTS

For many years, the use of competitive enhancement products, such as those defined as prebiotics, probiotics and competitive exclusion products have largely been restricted to use within the poultry industry as means of growth promotion (Callaway et al., 2008). With continued bacterial resistance to commonly used antibiotics globally, the sub-therapeutic use of these antimicrobial agents within food production systems have come under increasing spotlight, with ever stringent legislature now in place to reduce its usage (Patterson & Burkholder, 2003). As understanding behind the importance of the gut microbiota in health and disease of living organism continues to grow, the area of competitive enhancement products in livestock industries is rapidly evolving and attracting significant investment as a viable alternative to antibiotic use.

Microecology preparations can be employed in multiple different facets, most commonly ranging from prebiotics, defined Gibson & Roberfroid (1995) as 'non-digestible food ingredients that have a beneficial effect on the host by selectively stimulating already existing bacterial species' growth and/or activity' to probiotics, defined by WHO as 'live microorganisms which when administered in adequate amounts will confer a health benefit on the host' (FAO/WHO, 2001). While results of *in vivo* and *in-vitro* studies indicate efficacy of these pre- and probiotic formulations against *Campylobacter* infection of the broiler chicken (Ghareeb et al., 2012; Smialek et al., 2018), this opinion remains controversial. This variation in demonstrated effectiveness may be influenced by a multitude of factors, most notable being the exact source and compositional complexity of these pre- and probiotic formulations (Clavijo & Flórez, 2018). Following the observation that probiotic strains derived directly from chicken species were more effective in pathogen exclusion than those derived from alternate sources, use of a product representing a more 'natural' avian microbiota has been suggested as a more efficacious alternative (Clavijo & Flórez, 2018).

The prospect of introducing a 'normal' microbial population from a host animal to that of the GI tract of a recipient has been defined as 'competitive exclusion' treatment and largely stems back to a pioneering study performed in 1973 (Callaway et al., 2008). Here, Nurmi & Rantala (1973) presented preliminary result demonstrating how administration of adult chicken microbiota could reduce establishment of experimental *Salmonella* spp infection when given early post-hatch. Four decades later and this principle has led to the development and commercialization of multiple CE products for use within the poultry industry (Schneitz, 2005).

While differences exist between the mechanistics of production and exact composition, each intervention has been formed off the basis of derivation from the caecal content and/or caecal wall of domestic poultry (Schneitz, 2005). The first known commercially available CE intervention, BROILACT® (Orion Corporation, Espoo, Finland), was launched across Finland and Sweden in 1987, with subsequent development in 1994 to a lyophilized formulation (Nakamura et al., 2002; Schneitz & Hakkinen, 1998). Composed of 32 strict and facultative anaerobic bacterial types, BROILACT® is a highly defined mixed culture derived from a single healthy hen (Nuotio et al., 2013). Since its release, BROILACT® has reported success in reducing *Salmonella* colonisation of commercial chickens in field conditions, alongside tentative links to protection from *Campylobacter* spp. (Palmu & Camelin, 1997; Schneitz, 2005). However, continued large-scale use of BROILACT® has been somewhat restricted by a relatively short product shelf-life, allowing for the development of novel CE products with ability for extended periods of storage (Nakamura et al. 2002). Aviguard® (Lallemand, Worcestershire, UK) Is a freeze-dried product of fermentation launched in 1993 and applied to chickens and turkeys via drinking water or spray application (Abudabos, 2013; Nakamura et al., 2002). Unlike BROILACT®, Aviguard® contains a mixture of live bacterial strains from the caeca of an adult healthy SPF chicken, with this commensal microflora only being partially characterized (Nakamura et al., 2002; Schneitz, 2005). Aviguard® is marketed as a CE product mainly associated with the reduction in colonisation of *Salmonella* spp., however protection from pathogenic *E. coli* has been reported.

While the precise mechanistic action of CE interventions remains to be defined, effects are largely thought to be bacteriostatic by inhibiting the replication of intestinal pathogenic microorganisms within the caeca (Mead, 2000). While so much is yet to be discovered regarding avian pathogen interaction, alongside the microbe-microbe interactions of the intestinal tract, it is unlikely that we will truly understand the exact therapeutic actions behind these CE interventions for some time (Mead, 2000). However, five main modes of action have been postulated, described as (1) physical prevention of pathogen attachment and invasion, (2) nutritional competition direct and indirectly (3) production of biochemical antimicrobial products (e.g. SCFAs) (4) biological stimulation of the host immune system and (5) chemical reduction of the caecal pH creating a more hostile environment. With early studies (Soerjadi et al., 1981) stating protection of chicks from *Salmonella* colonisation as early as 1 – 4 hours after CE administration, it is largely considered that the physical process of direct competition between commensal and pathogenic microorganisms for attachment sites and nutrients is

likely to play an important protective role (Callaway et al. 2008; Mead 2000; Schneitz 2005). Physical competition processes are unlikely to form the sole processes by which CE therapies exert their effect, with pathogenic bacterial species with ecologies not requiring attachment to host epithelia also showing reduction in burden following CE exposure (Mead 2000). Production of SCFAs within the caecal crypt is also widely implicated in this protection through both direct and indirect means of reducing the competitive fitness of invading pathogenic bacteria (Callaway et al. 2008). Although clearly able to provide some degree of benefit to the commercial broiler chicken, this effect can often be transient allowing for subsequent infection at points of re-challenge (Cammarota et al., 2014). A practical basis for this limited establishment resilience could simply be due to initial exclusion product source and processing. With Aviguard® first isolated from SPF chickens before being fermented *ex-vivo*, crucial alterations in product composition could occur here, reducing the applicability as a truly 'natural' avian gut product.

CAECAL MICROBIOTA TRANSPLANT (CMT)

Fecal microbiota transplantation (FMT) refers to the transfer of a complete microbial environment from a healthy donor directly to recipient individual of the same species (Niederwerder et al., 2018). Unlike other microecological formulations described, FMT processes include the transfer of the complex fecal microbiota in its entirety, including bacteria, viruses, fungi, archaea, protozoa, metabolites and colonocytes (Bojanova & Bordenstein, 2016). This concept has led to the understanding that FMT is more than merely the transfer of beneficial microflora, but an entire organ transplantation in its own right (Borody & Khoruts, 2012). Previous work by Chapman et al. (2011) suggests that by increasing microecological preparation taxonomic complexity, an environment favoring establishment of the desired 'probiotic' taxa may be derived from other species much less in abundance, with these not going on to form strong colonisation footholds themselves. Since the caeca represents the intestinal region showing most dense microbial population within the chicken, this is the site of potentially most influence when considering donor microbiota for chicken microbiota transplantation (Sergeant et al., 2014). With the microbial taxonomic community structure significantly different between caecal and fecal content, use of caecal matter as donor material may be more representative of the 'natural' avian microbiota desired. As such, although this thesis will consider methodologies in regard to caecal microbiota transplantation (CMT) within the broiler chicken, published literature relating to this novel concept is negligible. For the purposes of this literature review, we will focus on concepts

relating to FMT, in the understanding that identified theory within such research will be conceptually and functionally applicable to CMT methods.

Although dating back to the 4th century, it is largely the clinical application of FMT in the treatment of human Clostridium difficile infection that has heralded its notoriety (Niederwerder et al., 2018). FMT application against antimicrobial non-responsive *C. difficile* infection was first described in 1983 and has since become highly successful as therapy administered across many mainstream hospitals (Niederwerder et al., 2018; Zeng et al., 2019). Alongside application to further human conditions including inflammatory bowel disease (IBD), Irritable bowel syndrome (IBS) amongst other conditions, FMT is widely utilized within veterinary medicine (Niederwerder et al. 2018). Described in terms of transfaunication, FMT provided a means of restoring normal rumination within cattle following digestive or metabolic disorders (Niederwerder et al. 2018). Over time, implementation of FMT therapy has emerged in small animal practice as a means of treating canine parvovirus infection in puppies alongside colitis in horses (Pereira et al. 2018).

While more complex in ecological composition, it is of largest inference that the mechanisms behind FMT function are simply an extension of the mechanisms described for CE based products described above. This being said, with the intestinal immune system showing great sensitivity to the microbial communities inhabiting both lumen and mucosal lining, the heightened complexity in infused transplantation microbiota might increase modulation of both innate and adaptive mucosal immune responses (Burrello et al., 2018). Studies by Hu et al. (2018) provides results on increase growth performance, intestinal barrier integrity and innate immune function in pigs orally gavaged with FMT therapy from donor pigs. Prophylactic use of FMT is more commonly associated with both porcine and poultry application, as oppose to therapeutic use described in most published literature associated with human use (Niederwerder et al., 2018). With the infused fecal material given to therapy recipients of an undefined nature, the reliance on highly selective donor selection cannot be understated. While exact donor exclusion criteria will depend on species and indication, common screening practices consider genetic background, phenotypic characteristics, infectious disease, common pathogens and other indications (Hu et al., 2018).

EARLY LIFE MICROBIOTA PROGRAMMING IN BROILERS

While rapid production cycles of the commercial broiler chickens hamper the implementation of some disease control strategies, largely those relying on string adaptive immune response mechanisms, the nature of this system appears to offer an unusual opportunity for microbial based control strategies (Rubio, 2018). Post-oviposition, there is no contact between parent and offspring, completely eliminating the crucial influence this early experience has on offspring microbiota development (Rubio 2018). As previously stated, this early microbiota is able to rapidly establish unhindered and create an intestinal environment beneficial to their own requirements and a driver for the early immune development (Baldwin et al., 2018). The consequence of unnatural poultry hatching systems result in chicks earliest environmental exposure being that of non-avian bacterial sources such as human workers and transport crates (Rubio, 2018).

The highly dynamic microbial variation is thought to exist only during early development, with Rubio 2018 stating continuation until 3 days post-hatch. The same work states clear differences in the at-hatch administration and of probiotic bacteria in the development of a stable, change resistant microbiota compared to natural environmental acquisition of the same bacterial taxa (Baldwin et al. 2018).

SAFETY OF MICROFLORA PRODUCTS WITHIN THE POULTRY INDUSTRY

Although modulation of the microbiota shows obvious promise in disease prevention, multiple areas of research have described increases in risk of disease associated with unnatural intestinal colonisation (Bartnicka et al., 2015). One of the largest safety concerns associated with CMT introduction into regular clinical practice is the complexity associated with between-batch standardization and characterization. With substantial variation in microbial communities between individuals, reproducing specific active components cannot yet be assured (Bartnicka et al., 2015). Introducing microecological therapies of relatively undefined nature raises crucial safety concerns regarding the introduction of potentially pathogenic taxa to recipient species (Bartnicka et al., 2015).

AIMS OF THIS THESIS

While our understanding of the complex nature of *Campylobacter* infection within the broiler chicken continues to evolve, there remains many gaps in currently available literature. Of

perceivably greatest importance to public health is the ability to reduce *Campylobacter* colonisation of the broiler chicken prior to point of slaughter. Here we use a collection of methodologies to first understand the ecology of *Campylobacter* infection within the broiler chicken, and subsequently, how frequency and severity of such infection can be minimized using novel microflora therapies.

Using *in vivo* experimental models, Chapter 2 aims to describe the infection dynamics of *Campylobacter* infection within the broiler chicken until the commonly used commercial point-of-slaughter. This understanding can be used to supplement the further analysis of innate, adaptive and regulatory immune mechanisms associated with key time points post-infection.

Chapter 3 will form the initial proof-of-concept for the efficacy of CMT use within broiler chickens against *Campylobacter* infection at a flock level. Here we hope to define a basic mechanism of administration and outline the effects of this therapy on expressed chicken phenotype and disease resistance.

As a progression from the preceding chapter, Chapter 4 both *in vivo* and *in-vitro* experimental protocols to uncover both the efficacy and potential immunological mechanisms of CMT action. With similarly derive CE products currently available but relatively seldom understood, direct comparisons will be drawn between the action of CMT and a commercial microflora product, Aviguard®.

Finally, Chapter 5 will use genomic 16S rRNA techniques on the caecal samples taken from *in vivo* protocols of Chapter 4 to provide a basis of understanding in how both CE and CMT therapies work to modulate the chicken microbiota at early time points prior to infection. We can then draw comparison between these alterations in microbial structure and composition and relate them to the expression of reduced susceptibility to *Campylobacter* colonisation

Chapter Two: A study into the dynamics of prolonged infection of the broiler chicken with *Campylobacter jejuni*

INTRODUCTION

Campylobacter continues to be the most common cause of bacterial foodborne gastroenteritis worldwide. Epidemiological reporting reports 250,161 laboratory confirmed cases of campylobacteriosis across 29 EU/EEA countries in 2017, marking a slight decrease compared to 2016 (ECDPC, 2019). Data on the prevalence of campylobacteriosis outside Europe and North America remains relatively sparse, however it is likely that infection is endemic in Africa, Asia and the middle east (Kaakoush et al., 2015). Although incidence varies vastly from country to country, it is generally understood that true disease incidence is much higher than that recorded, largely due to under-detection and under-reporting (Wagenaar et al., 2013). Wagenaar et al. (2013) estimated that from the 198,252 EU campylobacteriosis cases reported in 2009, the true incidence of disease was 46.7 times this value, exceeding 9 million cases. Furthermore, the economic burden to the EU of *Campylobacter* infection is thought to be in excess of € 2.4 billion per year in lost productivity (European Food Safety Authority, 2014). Of even greater concern is the prevalence of campylobacteriosis in the developing world, particularly amongst children < 5 years of age within these regions. One study reported infection rates as high as 60,000 per 100,000 population (Hanif et al., 2018). Poor sanitation, close contact with live animals and inefficient cooking of poultry meat are considered the key contributions to the hyperendemic nature of infection within the children of these countries (Zilbauer et al., 2008).

Infection of *Campylobacter* in humans is largely associated with two reservoirs of infection; infected animals and birds and infected water sources. Links between untreated water and human campylobacteriosis are well established (Wilson et al., 2008). However, the contribution to disease resulting from this bacterial reservoir appears sporadic and highly seasonally dependent (Wilson et al., 2008). As such, infected water sources are thought to be a lesser public health risk, particularly in countries with a more developed sanitation infrastructure (Lin, 2009). Wilson et al. (2008) goes on to state that wild animal and environmental *Campylobacter* reservoirs are responsible for an estimated 3% of human campylobacteriosis cases, with these cases also peaking during warmer summer months. Regarded as the most important source of transmission, is the zoonotic transfer of *Campylobacter* from a domesticated animal host. *Campylobacter* has been isolated from the gut of multiple warm-blooded farm, wild animal and bird species (de Zoete et al., 2007). Despite this wide repertoire of hosts, chickens continue to be the largest source of infection, contributing to 50 - 80% of human campylobacteriosis cases (BIOHAZ, 2011). Furthermore, up

to 30% of cases could be solely attributed to the handling, preparation and consumption of broiler chicken meat (BIOHAZ, 2011).

Despite a reported reduction in overall meat consumption across the EU over recent years, the poultry meat industry has continued to see production increase (BIOHAZ, 2011). Offering consumers, a cheap, lean and easily accessible source of protein has led to increased poultry meat consumption across countries both within and outside of Europe. With chicken being a natural vector of *Campylobacter* infection and estimated positivity within EU flocks sometimes reaching 90%, the *Campylobacter* reservoir harbored within the poultry industry confers a major public health concern (de Zoete et al., 2007). It is reasonable to believe that reduction in both the incidence and the level of *Campylobacter* contamination in commercial poultry meat would result in a considerable reduction in human campylobacteriosis. The European Food Safety Authority (EFSA), based on work by Rosenquist et al. (2003), predicts that half of all cases of human campylobacteriosis within the EU could be eliminated by reducing *Campylobacter* positivity within poultry flocks to 25 % per member state. Further to this, using the Rosenquist et al. (2003) risk assessment framework encompassing hazard identification, hazard characterisation, exposure assessment and risk characterisation on Dutch campylobacteriosis cases, it was suggested that simply reducing poultry carcass infection load by 100-fold could result in 30 times less campylobacteriosis incidence (Lin, 2009; Rosenquist et al., 2003). This prediction of the effects of specific mitigation strategies on the incidence of *Campylobacter* infection within humans has been used to inform both the Food Standards Agency (FSA) and EU policy on potential *Campylobacter* controls (FAO, 2009).Controlling infection within commercial poultry flocks is made more complex by the fact that broiler chickens rarely show obvious clinical signs of *C. jejuni* infection, despite levels of colonisation reaching an excess of 10^{10} CFU/g of caecal material (Ghunaim, 2009).

Although more than 20 separate species of the *Campylobacter* genus have been identified within humans, the two species known to be the predominant cause of human campylobacteriosis are *C. jejuni* (~ 90%) and *C. coli* (~ 10%) (de Zoete et al., 2007) and therefore this study will primarily focus on *C. jejuni.* Colonisation of commercial poultry flocks with *C. jejuni* generally begins from 2-3 weeks of age, although successful colonisation models have been described following experimental infection at a younger age (Sahin et al., 2002). Understanding of this apparent lag phase in infection remains limited, although many facets of opinion exist, from the presence of maternally derived antibodies to intestinal microbial

flora transition (Sahin et al., 2002). Once present within a flock, *C. jejuni* is able to rapidly and effectively colonise almost all birds (>95%) within only a period of several days (Hermans et al., 2014). The main site of *C. jejuni* colonisation within its avian host is the intestinal tract, with highest numbers primarily found at the distal end of the gastrointestinal tract (GIT) within the bind caecal crypts (Humphrey et al., 2014). Coward et al. (2008) found that within only 24 hours of ingestion, *C. jejuni* was able to migrate to the caeca, multiply and establish a strong colonisation from only 35 CFU. Once established within the caecal crypts, *C. jejuni* can be found persistently within the caecal lumen and mucosal layer without adhering or invading the intestinal epithelial cell layer (de Zoete et al., 2007). Colonisation of the caeca shows little tendency towards clearance and as such, persistent colonisation is exhibited that extends until slaughter (Hermans et al., 2014). Colonisation of the avian intestinal tract has long been thought of as commensal in nature, lacking host associated intestinal inflammation and pathology (Mead, 2000). Such a concept has been queried over recent years, with Humphrey et al. (2014) proving *C. jejuni* colonisation leads to diarrhoea alongside compromises in the integrity of the mucosal epithelial barrier (Awad et al., 2017). Concomitantly, *C. jejuni* spread beyond the confines of the caeca has been observed, with bacteria recovered from the small intestine, crop, gizzard, liver and spleen (Chaloner et al., 2014). This ability to cause systemic infection has put further question onto the apparent benign infection model previously thought to be established by *C. jejuni* within avian hosts.

With evidence that *C. jejuni* is able to traverse the intestinal epithelial barrier and enter systemic circulation the close interaction between bacterium and host suggested by this would likely induce local and systemic immune responses (Han et al., 2016; Smith et al., 2008). While little published evidence exists describing the nature of an innate immune response to *C. jejuni* within the chicken, some description of humoral response to infection is available (Lacharme-Lora et al., 2017). It has been suggested by Lacharme-Lora et al. (2017), that although immune clearance of *C. jejuni* may be somewhat attributed to antibody production within birds older than average commercial slaughter age, antibody-dependent clearance is negligible in birds younger than 8-weeks of age. Such clearance was also found to be largely localized within the ileum and jejunum, with limited influence within the caeca, implying antibody production in these more proximal gastrointestinal tract (GIT) sites will only prevent *C. jejuni* recolonisation locally, not affecting subsequent colonisation in more distal sites such as the caeca (Lacharme-Lora et al., 2017).

To induce the adaptive immune responses discussed, it is first necessary for *C. jejuni* to elicit some degree of innate immune response (Smith et al., 2005). Much of the work characterizing these pathways have been developed *in vitro* using avian cell lines (Smith et al., 2005). However, observably distinct invasion capabilities are associated with use of *C. jejuni in vitro* using avian enterocyte lines (Smith et al., 2005). As such, although studies have made advances in identifying differential expression of immune transcripts associated with *C. jejuni* infection using these models, such data may not be truly reflective of an *in vivo* system. Of note is the work conducted by Reid et al. (2016), who utilized *in vivo* experimental challenge designs to assess time-dependent cytokine response to *C. jejuni*. Here, they highlight the overarching dominance of protective Th17 responses, such as IL-1ß and IL-6 in the stimulation of IL-17A. Other factors, such as those associated with T regulatory (Treg), pathways have also been investigated for their role in maintaining broiler chicken intestinal homeostasis (Humphrey et al., 2014).

In order to enhance our current ability to control *C. jejuni* infection within commercial poultry industry worldwide, it is first essential to understand the biological infection dynamics and responses to infection within its natural avian host. With chicken being both a reservoir of *C. jejuni* and an important food source for humans across both the developing and developed world, *in vivo* poultry infection models act as tools in understanding aspects of host colonisation (Young et al., 2007). From this foundation in understanding, we would be more able to identify potential control strategies and when these should be implemented; either prior to or within the stages of *C. jejuni* colonisation.

The aim of both experiments 1 and 2 within this study were to conduct *in vivo* poultry infection trials that would demonstrate the infection dynamics and host immune response to *C. jejuni* following a longer period of infection than that generally studied. From this we were hoping to elucidate when *C. jejuni* colonisation occurs within the small intestine and the caeca and whether such colonisation status would influence the profile of immune response generated. We were also looking to identify if extra-intestinal spread to the liver and spleen occurred within broiler chickens and whether this was correlated with level of *C. jejuni* load within the GIT of these birds.

MATERIALS AND METHODS

BACTERIAL ISOLATES AND GROWTH CONDITIONS

Strain *C. jejuni* M1 was selected for use within this study, as it has a well characterized genetic background within published literature and is a strain associated with transmission from poultry to humans (Chaloner et al., 2014; Humphrey et al., 2014). *C. jejuni* M1 is of sequence type (ST) 137 (clonal complex [CC] 45) human isolate kindly provided by Lisa Williams (University of Bristol) (Chaloner et al., 2014). This ST is commonly represented within UK retail poultry and has been used within our laboratories for similar *in vivo* methodologies (Humphrey et al., 2014).

C. jejuni stock was maintained at - 80 °C on Microbank™ beads (ProLab Diagnostics, Cheshire, UK) until use. Stored *C. jejuni* strains were subsequently cultured using a 5 µl sterile disposable loop as previously described by (Chaloner et al., 2014). Briefly, bacteria were grown on Columbia blood agar (CAB) (Lab M Ltd., Heywood, Lancashire, UK) supplemented with 5% defibrinated horse blood (Oxoid, Basingstoke, Hampshire, UK at 41.5 °C for 48 hours under microaerobic conditions (80 % N_2, 12 % CO_2, 5 % O_2 and 3 % H_2).

A single colony from the grown bacterial culture was selected and used to inoculate 10 ml of Mueller Hinton broth (MHB) (Lab M Ltd, Heywood, Lancashire, UK) in a sterile 30 ml universal tube. Isolation of a single colony from the initial growth minimised the possibility of genetic variation within the resulting *C. jejuni* working solution. The 10 ml working liquid culture was grown for 24 hours under microaerobic conditions at 41.5°C with a loosely capped lid. After overnight incubation, liquid culture was mixed using a vortex mixer and 2 ml of culture tested for Optical Density (OD) value using a spectrophotometer (Cecil CE2040, Cambridge, UK). Measured OD of the liquid culture was adjusted to 0.1 - 0.13 at OD_{600}, corresponding to a count of 1 x 10^8 CFU/ml. To obtain the desired 1 x 10^6 CFU/ml for subsequent infection, a 1:200 (v/v) dilution of 100 µl liquid culture into 20 ml MHB was made. Serial 10-fold dilutions of the final liquid culture in 1 x Maximal Recovery Diluent (MRD) (Lab M Ltd, Bury, UK) were made to 10^{-8} for viable colony enumeration via the Miles & Misra method (Miles & Misra, 1938). Briefly, two agar plates were visibly divided into 8 equal sectors (four sectors per plate) and thoroughly dried. In each sector, 3 x 20 µl of the appropriate sample dilution was pipetted onto the surface of the agar, with these agar plates being left upright on the bench to dry

before incubation. Each sector was then observed for colony growth. Colonies were enumerated in the highest dilution series that contained three replicates of full-size, discrete colonies. Ideally each individual replicate within a sector will contain between 2 - 20 colonies.

EXPERIMENTAL ANIMALS

Experimental infection trials were carried out at the University of Liverpool poultry unit (Liverpool, UK) in accordance with United Kingdom legislation governing experimental animals. All work was conducted under project licences PPL 40/3652 (Experiment 1) and P999B8C93 (Experiment 2) and was approved by the University of Liverpool ethical review process prior to the award of the licence. All animals held at the site were checked a minimum of twice daily to ensure individual and flock animal health and welfare. All *in vivo* experiments used day-old broiler chicks (Ross 308) of mixed sex, obtained from a local commercial hatchery. Ross 308 remains the most commonly reared broiler breed within the UK, justifying its use within these experimental models. All chicks were transported directly from the hatchery environment to the experimental unit and observed for any potential indications of ill health.

Chicks were maintained according to treatment group in separate experimental rooms within floor pens at a stocking density in accordance with Home Office Code of practice recommendations. All rooms were supplied with filtered air supply, while groups intended for experimental infection protocols were housed in rooms with lobbied entry and additional dedicated protective clothing and boots. All animals were housed in conditions previously described by Humphrey et al. (2014). Birds were given *ad libitum* access to water and a pelleted vegetable protein-based diet (SDS, Witham, Essex, UK). Feeders and drinkers were provided at a level of 1 per 15 birds. Room temperature was kept at 30^{o}C before being reduced to 20^{o}C when the birds were three weeks of age. To limit welfare problems associated with wet litter and to limit within-group retransmission, litter was changed, and pens were cleaned once every four days.

At 14 d.p.h (days post hatch)*, Campylobacter* negative status was confirmed for all birds prior to experimental infection through cloacal swabbing, with full description of swabbing procedures listed in Chapter 3. Swabs were subsequently streaked onto *Campylobacter*-selective blood-free agar, (modified charcoal-cefoperazone-deoxycholate agar [mCCDA]) supplemented with *Campylobacter* enrichment supplement (SV59; Mast Group Ltd, Bootle,

Merseyside, UK), covering the entire agar surface before incubating at 41.5°C for 48-hours in microaerobic conditions.

At 21 d.p.h, all birds within the challenge group of each experimental trial were orally challenged with 0.2 ml 10^6 CFU/ml *C. jejuni* M1 in MHB, prepared as previously described. All birds within the control group were administered 0.2 ml of sterile MHB. Challenge at 21 d.p.h has previously been shown as a robust model of infection that mimics field infection within the UK, where birds typically become infected around three weeks of age (Sahin et al., 2003). Inoculation material was administered through oral gavage using a sterile 1 ml syringe (Fisher Scientific, Loughborough, UK) and a custom produced sterile gavage needle. The bird's neck was gently stretched upward and the beak held open. The gavage needle was placed gently into the oesophagus and the plunger depressed to dispense 0.2 ml of either sterile MHB or *C. jejuni* inoculation material. All birds were observed closely for 2 hours post infection.

EXPERIMENTAL TRIAL DESIGNS

This study is comprised of two separate experimental trials completed in accordance with protocols ZIPP 41 and ZIPP 56. Figure 2 & 3 provide a visual explanation of key experimental features and timelines for experiment 1 and experiment 2.

Experimental design – Experiment 1

Age-matched, 1 d.p.h mixed sex Ross 308 chicks (n = 90) were introduced to the University of Liverpool high-biosecurity poultry unit under housing conditions described previously. On point of entry, the chicks were randomly assigned to one of two groups; Group 1 (n=57) or Group 2 (n = 30).

Prior to infection, at 14 d.p.h, all animals were confirmed to have *Campylobacter* negative status as previously described. At 21 d.p.h, all birds within the *C. jejuni* Group 1 were orally infected with 0.2 ml 10^6 CFU/ml *C. jejuni* in MHB via oral gavage. All birds in Group 2 were given 0.2 ml sterile MHB via oral gavage. Preparation of inoculum and infection protocols were conducted as described previously. From this point, experimental groups 1 and 2 were named *C. jejuni* infected and non-infected control groups respectively.

At 23 (2 days post infection [d.p.i]), 28 (7 d.p.i), 35 (14 d.p.i), 42 (21 d.p.i) and 49 (28 d.p.i) days post hatch, randomly selected birds were culled via cervical dislocation from the *C. jejuni* infected group (see Figure 2) and non-infected trial group (n = 6). Blood samples were collected via cardiac puncture immediately post-cull, before samples of splenic & liver tissues and caecal & ileal content were aseptically collected. An additional ileal tissue section was collected, and ileal content removed for subsequent gut wash processing.

Experimental design – Experiment 2

Age-matched, 1 d.p.h mixed sex Ross 308 chicks (n = 55) were introduced to the University of Liverpool high-biosecurity poultry unit under housing conditions described previously. On point of entry, the chicks were randomly assigned to one of two groups; Group 1 (n=26) or Group 2 (n = 27).

Prior to infection, at 14 d.p.h, all animals were confirmed to have *Campylobacter* negative status as previously described. At 21 d.p.h, all birds within the *C. jejuni* Group 1 were orally infected with 0.2 ml 10^6 CFU/ml *C. jejuni* in MHB via oral gavage. All birds in Group 2 were given 0.2 ml sterile MHB via oral gavage. Preparation of inoculum and infection protocols were conducted as described previously. From this point, experimental groups 1 and 2 were named *C. jejuni* infected and non-infected control groups respectively.

At 23 (2 days post infection [d.p.i]), 28 (7 d.p.i), 35 (14 d.p.i) and 42 (21 d.p.i) days post hatch, a pre-defined number of randomly selected birds from each group were culled via cervical dislocation (Figure 3). Blood samples were collected via cardiac puncture immediately post-cull, before samples of splenic & liver tissues and caecal & ileal content were aseptically collected.

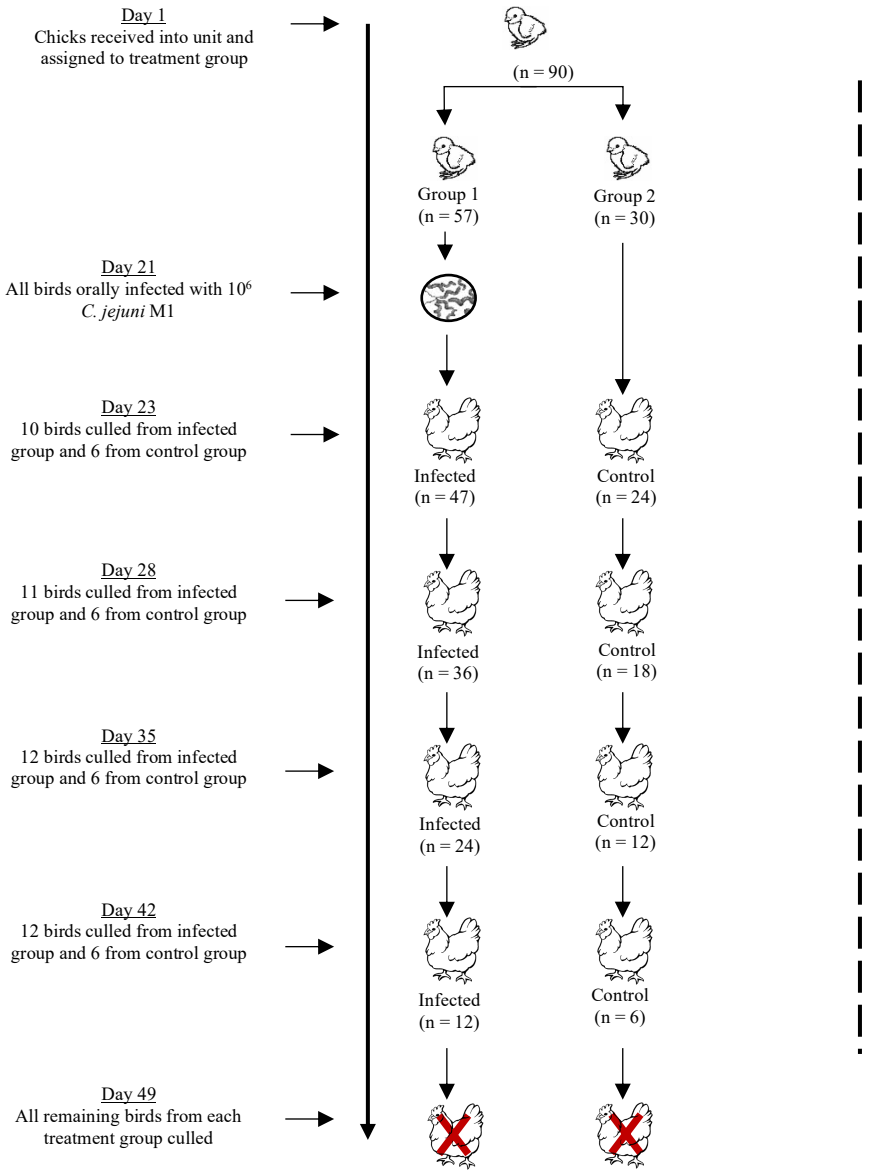


Figure 2. Key time points associated with experimental trial 1

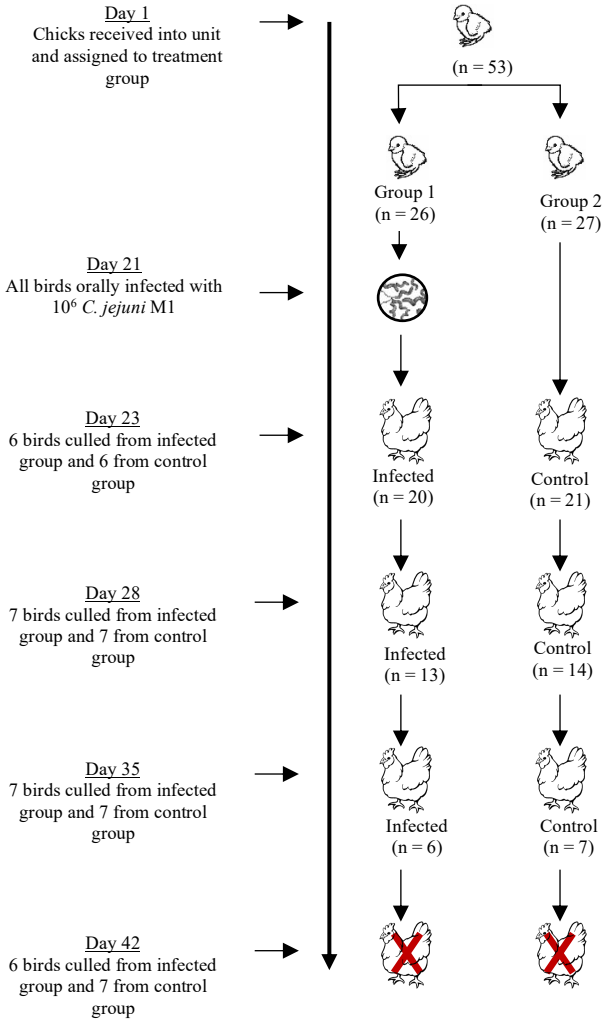


Figure 3. Key time points associated with experimental trial 2

POST-MORTEM SAMPLING

Birds were culled according to a pre-determined protocol specific to each experiment. All tubes pertaining to samples for bacteriological analysis were weighed ante-mortem. Control groups always underwent post-mortem sampling prior to infected groups to avoid cross contamination. Dissection kits (forceps and scissors) were sanitised by returning to a 100 % EtOH bath following each use and new kits were used between different experimental groups. Birds were euthanized by cervical dislocation before being surface sterilised with 70 % EtOH. Sterile latex gloves were used and changed for each bird, and then again after initial bird skin removal. All consumables used during post-mortem analysis can be seen in Table 3 below. Post-mortem sampling was conducted according to the following order of process:

1. Using sterile forces and scissors, the skin and muscle covering the chest cavity was removed and the heart and liver exposed.
2. A cardiac puncture was performed using a sterile 23G gauge needle and a sterile 2 ml syringe to obtain ~ 0.5 ml – 1 ml of whole blood.
3. A 1-3 gram sample of liver was isolated for bacteriological analysis.
4. The bird was turned onto one side and the spleen located and removed. Half of the spleen was collected for bacteriological analysis.
5. The large intestine was cut at the junction immediately above the cloaca and the small intestine cut immediately below the duodenum to free the section of the gastrointestinal tract from the body cavity. The intestines were retained, and the carcass disposed of. The following samples were obtained;
    - 5.1. A small section of the ileum was cut, and the contents collected for microbiota analysis.
    - 5.2. A second small (~ 20 cm) section of ileal tissue (content removed) was collected and place in a sterile Falcon tube for ELISA analysis.
    - 5.3. One caecal tonsil, a visible nodule of lymphoid tissue located at the proximal end of the blind ended caeca, was removed and placed in 1 ml RNA later (Sigma, Poole, Dorset, UK).
    - 5.4. Caecal contents were then collected for bacteriological analysis.
    - 5.5. A small section of caecal tissue (contents removed), ~0.5 cm in length, was removed and placed in 1 ml RNA later.

All blood samples collected during post-mortem analysis were centrifuged at 13000 x*g* for 5 minutes. The serum was removed and stored in a sterile 2 ml Eppendorf at -20°C for subsequent ELISA analysis.

Table 3. Complete list of consumables used according to sample type

Consumable	Sample
1 x 2 ml Eppendorf / sample 1 x 23 g Needle / sample 1 x 2 ml Syringe / sample	Blood sample
1 x 30 ml Universal / sample (weighed)	Bacteriology (caecal content, ileal content, liver tissue, spleen tissue)
1 x 2 ml Eppendorf / sample containing 1ml RNA later	RNA extraction (caecal and caecal tonsil tissue)
1 x Dissection kit / treatment group Latex gloves Ethanol White roll Clinical waste bags	General

BACTERIOLOGY

At post-mortem, samples of spleen, liver, caecal and ileal content were taken for bacteriological analysis. Samples outside of the GIT were collected first to avoid contamination from intestinal contents. All samples were aseptically collected and placed into separate, pre-weighed 30 ml universal tubes, which were then re-weighed following sample addition.

To spleen and liver samples, a 1 x MRD (Lab M Ltd., Heywood, Lancashire, UK) in 1:5 dilution was added. Diluted tissue samples were subsequently homogenised in a MicroStomacher 80 (Seward, UK) for 1 minute. Following 15 seconds of vortexing, 100 µl of sample homogenate was plated onto mCCDA agar, ensure sample was spread to cover the entirety of the agar surface using Fisherbrand™ L-shaped cell spreaders (Fisher Scientific, Loughborough, UK). The plates were incubated at 41.5°C for 48 hours in microaerobic conditions. A further 200 µl of homogenised sample was used to inoculate 2 ml of Exeter selective enrichment broth (1100 ml nutrient broth, 55 ml lysed defibrinated horse blood, *Campylobacter* enrichment

supplement SV59 [containing trimethoprim (10 mg/L) and amphotericin B (2mg/L); Mast Group Ltd, Bootle, UK] and *Campylobacter* growth supplement SV61 [containing sodium pyruvate (250mg/L), sodium metabisulphate (250 mg/L) and ferrous sulphate (250mg/L); Mast Group Ltd]) and incubated at 41.5°C for 48 hours in microaerobic conditions. Following enrichment, samples were vortexed and plated onto mCCDA using a 3 μl loop. All plates were incubated at 41.5°C for 48 hours in microaerobic conditions before being assessed for *C. jejuni* growth.

To caecal and ileal content, a 1 x MRD in 1:10 dilution was added. Diluted contents were subsequently vortexed for 1 minute at maximal speed to ensure full sample dispersal within diluent. Each sample was serially diluted in 1 x MRD to 10^{-8} and plated onto mCCDA agar using Miles and Misra methodologies as previously described (Miles & Misra, 1938). All plates were incubated at 41.5°C for 48 hours in microaerobic conditions before being enumerated.

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism version 7.00 for Mac OS X (GraphPad Software Inc., San Diego, USA). Prior to further statistical analysis, all data was first assessed for distribution normality using D'Agostino-Pearson omnibus normality testing. Pairwise treatment group comparisons of normally distributed data sets ($p > 0.05$) were conducted using an Unpaired *t*-test and described using data mean and standard deviation values (SD). Pairwise treatment group comparisons of non-normally distributed data sets ($p < 0.05$) were conducted using a Mann Whitney-U test and described using data median and interquartile range (IQR). Statistical significance was determined using a $p < 0.05$ threshold. For statistical comparisons assessing more than two distinct groups, Kruskal-Wallis testing was used with a $p < 0.05$ threshold.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Production of C. jejuni whole cell lysate antigen

Frozen stocks of *C. jejuni* M1 stored at – 80 °C were grown on CBA at 41.5°C for 48 hours under microaerobic conditions. A single colony from the grown bacterial culture was selected and plated onto CAB, covering as much of the agar surface as possible. This was repeated a further

three times to create four CAB plates before incubation at 41.5°C for a further 48 hours under microaerobic conditions.

Following incubation, the agar surface of each plate was flushed with approximately 1.5 ml sterile 1 x PBS (Lab M Ltd, Heywood, Lancashire, UK), using a sterile disposable 5 µl loop to manually disrupt bacteria from the agar surface. This bacterial suspension was collected and centrifuged at 4000 x *g* for 20 minutes. The supernatant was aseptically removed using a pipette and the remaining bacterial pellet re-suspended in 1.5 ml sterile 1 x PBS. Bacterial suspensions were incubated in a water bath at 65 °C for 5 hours. Bacterial suspensions were subsequently sonicated 5 freeze thaw cycles in liquid nitrogen, ensuring complete thaw of bacterial suspensions between each cycle. Bacterial suspensions were centrifuged at 4000 x *g* for 20 minutes at 4°C before all supernatant was removed aseptically using a pipette and pooled in a sterile ultracentrifuge tube. The supernatant was then ultra-centrifuged at 30000 x *g* for 20 minutes at 4 °C before the supernatant was aseptically removed using a pipette, placed into sterile 2 ml Eppendorf tubes and stored at – 20 °C until further processing.

Protein concentration was subsequently quantified using a Pierce ™ Modified Lowry Protein Assay kit (Product number #23240; ThermoFisher Scientific, Loughborough, UK), according to manufacturer's instructions. Briefly, 0.2 ml of Bovine Serum Albumin (BSA) (Sigma, Poole, Dorset, UK) standards of known concentration (ranging from 1 µg/ml – 1500 µg/ml) were prepared according to manufacturer's instruction. At 15 – second intervals, 1 mL of Modified Lowry Reagent (provided) was added to each standard alongside three replicates of our sample antigen of unknown concentration. All samples were vortexed and incubated for 10 minutes at room temperature (RT). Immediately following incubation, 100 µl of prepared 1 x Folin-Ciocalteu Reagent (provided) was added to each sample, vortexed and incubated for a further 30 minutes. Standard and sample optical density at 750 nm (OD_{750}) was measured using a spectrophotometer and a standard curve plotted to interpolate the protein concentration of our unknown antigen samples.

Serum IgY and IgM

Chicken serum Immunoglobulin M (IgM) and IgY were determined for blood samples collected at post-mortem using ELISA's, according to protocols previously described by Lacharme-Lora et al. (2017). In brief, Nunc-Immuno ™ MicroWell™ Flat-bottomed 96-well plates (Sigma,

Poole, Dorset, UK) were coated with 100 µl per well of *C. jejuni* whole cell lysate antigen, diluted in carbonate-bicarbonate buffer (pH 9.6) (Sigma, Poole, Dorset, UK) to a concentration of 10 µg/ml and incubated overnight at 4°C. Following incubation, plates were washed three times with PBS Tween-20 (0.05 %), (Sigma, Poole, Dorset, UK) before incubation with 100 µl per well blocking buffer for 1 hour at 37°C (0.05 % Tween-20 in PBS and 3 % skimmed milk powder). Plates were then washed once with PBS Tween-20 (0.05 %). Collected serum samples were diluted in blocking buffer for the detection of IgY and IgM. Plates were incubated with 100 µl per well (in duplicate) of the diluted serum samples for 1 hour at 37°C and washed three times in PBS Tween-20 (0.05 %). Specific anti-*Campylobacter* antibodies were detected by the addition of 100 µl per well alkaline phosphatase conjugated to either goat anti-chicken IgY (1:1000) or IgM (1:1000) (Serotec, Oxford, UK) diluted in blocking buffer, for 1 hour at 37°C. Plates were washed once further with PBS Tween-20 (0.05 %) and incubated with 100 µl per well of *p*-nitrophenyl phosphate (Sigma, Poole, Dorset, UK) in the dark for 30 minutes at RT. The reaction was stopped with the addition of 100 µl per well of 3N sodium hydroxide (Fisher Scientific, Loughborough, UK) before absorbance was determined using a microplate reader at 405 nm. A full experimental protocol is provided in Appendix 1.

All assays were conducted alongside duplicate 'blank' wells containing dH_2O in replacement of query sample. Resulting absorbances for all wells were first corrected for background absorbance by subtracting the mean 'blank' absorbance reading for each assay from all sample absorbance readings. Positive and Negative controls were included, in duplicate, for every assay plate. Control serum samples were sourced from the study described by Lacharme-Lora et al. (2017). The positive control sample confirmed correct technical implementation of the ELISA assay, being a blood serum sample from a *C. jejuni* infected experimental Ross 308 chicken with known absorbance values at OD_{405}. Blood serum used as a negative control was obtained from an experimental Ross 308 chicken successfully bursectomised by daily intramuscular injection of 3mg cyclophosphamide during four days post-hatch. The limit for positivity was determined at the level of such negative control.

Secretory IgA

As a means of determining secretory IgA levels within the ileum, a section of ileal tissue (without ileal content) was aseptically collected at post-mortem. Ileal sections were flushed with 10 ml m sterile 1 x PBS while the tissue was manually massaged. The flush was collected

and centrifuged for 10 minutes at 500 x *g* before aseptic collection of the supernatant with a pipette. All processed samples were stored at – 20 °C until further processing.

Quantification of secretory IgA within the processed samples was then conducted using an IgA Chicken ELISA Kit according to manufacturer's instructions (ab15, Abcam®, Cambridge, UK). Briefly, 100 µl of pre-prepared standard solutions (concentrations ranging from 12.5 – 400 ng/ml) including blank control (consisting only of provided diluent solution) were added in duplicate into pre-designated wells of a provided 96-well plate. Gut wash samples were diluted 1:5000 in 1 x diluent solution provided and added to wells of the provided 96-well plate in duplicate. Plates were incubated for 20 minutes at RT before being washed 4 times with 1 x wash buffer (provided). 100 µl of 1 x enzyme-antibody conjugate (provided) was added to each well and incubated for 20 minutes at RT in the dark. Plates were washed 4 times with 1 x wash buffer (provided) and 100 µl of TMB substrate solution (provided) was added to each well before incubation in the dark at RT for 10 minutes. Absorbance was determined using a microplate reader at 405 nm and a standard curve plotted to interpolate total secretory IgA concentrations in ng/ml

RNA EXTRACTION

Tissue samples of spleen, caeca, caecal tonsil and ileum were collected from all infected and control birds of experiment 1 and stored in 1 ml RNA*later*™ at -20°C until further processing (Sigma, Poole, Dorset, UK). Total RNA was extracted from 20 – 30 mg of all tissue samples using an RNeasy minikit (Qiagen, West Sussex, United Kingdom) according to manufactures instructions. Prior to extraction protocols, Buffer RLT solution (provided) was first supplemented with 10 µl of β- mercaptoethanol (Sigma, Poole, Dorset, UK) per 1 ml Buffer RLT. All tissues were disrupted using a TissueLyser (Qiagen, West Sussex, UK) at a frequency of 10,000/S for 10 minutes, with the addition of one stainless steel metal bead per sample. Isolated RNA was eluted into 50 µl of RNase-free water and stored at - 80 °C until processing. Total RNA yield per sample was determined using a Nanodrop (ND-1000) spectrophotometer, with samples showing low yield being re-extracted.

IMMUNE GENE EXPRESSION BY QUANTITATIVE REAL-TIME PCR

Cytokine and chemokine $2^{-\Delta\Delta}C_t$ RT-qPCR

mRNA expressional changes for the cytokines interleukin-1β (IL-1β), IL-4, IL-6, IL-10, IL-17A, Transforming growth factor ß$_4$ (TGFβ$_4$) and the chemokine ligand CXCLi2 were measured in these tissue samples by real-time quantitative reverse-transcription PCR (RT-qPCR) using a Rotor-Gene Q version 2.3.1.49 (Qiagen, West Sussex, UK) as previously described by (Humphrey et al., 2014). All primer and probe sequences for these genes have previously been described by (Shini & Kaiser, 2009) and are listed in Table 4 alongside threshold values used. Selection of cytokine, chemokine and growth factor genes for analysis was based upon demonstration of potential modulation within the chicken following *C. jejuni* infection. Reid et al. (2016) highlights particular importance of IL-1β and IL-6 in the upregulation of IL-17A, a pathway of suggested to have a protective response against *C. jejuni*. With Th17 cells known to have protective effects at mucosal barriers through their role as sentinels, CXCLi2 was considered as an important mediator to this pathway (Connerton et al., 2018). Connerton et al. (2018) goes on to state greatly increased IL-17A response following *C. jejuni* infection however no observed increase in caecal IL-10. This is in contrast to the work by Humphrey et al. (2014) who present differential expression of IL-10 within broiler chicken breeds following infection, emphasising its importance in the outcome of infection. Maintenance of gut homeostasis by IL-10 is often considered to be supplemented by the effects of TGFβ. While this represents on a subset of the cytokine repertoire that have been described as effectors following *C. jejuni* infection of the chicken, primer and probe sequences for these particular genes were readily available and demonstrated reaction profiles conducive to our tissue samples. For all reactions, 28S rRNA was used as the housekeeping gene to normalise mRNA levels between samples. All RNA samples were first diluted 1:10 using RNase-free water to obtain desired concentration per sample.

One-step RT-qPCR was performed using the RotorGene Probe RT-PCR kit (Qiagen, West Sussex, UK) to a final reaction volume of 20 µl. All reactions contained 1 µl of total RNA (at a concentration of 20 ng/µl), 10 µl of RotorGene Probe RT-PCR master mix, 0.2 µl of RotorGene reverse transcriptase enzyme mix, 1.6 µl forward primer (at 10 µM), 1.6 µl reverse primer (at 10 µM), 0.8 µl of probe (at 5 µM) and 4.8 µl of RNase-free water (Humphrey et al., 2014). Each sample was assessed in triplicate, with no-template control samples, containing RNase-free

water in place of total RNA, being used for each run. All reactions were conducted according to the following reaction profile listed in Table 4.

Table 4. Details of RT-qPCR amplification conditions using TaqMan PCR

	Step	Time	Temperature
	Reverse Transcription	10 minutes	50°C
	Enzyme Activation	5 minutes	95°C
Cycling X 40	Denaturation	5 seconds	95°C
	Data Collection	10 seconds	60°C

Mucin2 $2^{-\Delta\Delta}C_t$ RT-qPCR

mRNA expressional changes in the glycoprotein Mucin2 (MUC2) was measured in caecal tissue biopsy samples by RT-qPCR using a Rotor-gene Q version 2.3.1.49 (Qiagen, West Sussex, UK) as described previously (Humphrey et al., 2014). MUC2 is one of the most prominent mucin gel-forming mucin in the small and large intestine (Jiang et al., 2013). While MUC5AC and MUC6 are also widely utilised within published research as mucin's associated with prevention of pathogenic enteric disease in humans, primer sequences selected for these genes showed abnormal reaction profiles when assessed using our samples and so were not considered for this study. Primer sequences used are listed in Table 6 alongside the respective threshold value. For each reaction, β-actin rRNA (ACTB) was used as the reference gene to normalise mRNA levels between samples. Threshold values for each gene transcript were determined at 10 % of the curve plateau of known *Campylobacter*-positive control samples. All RNA samples were first diluted 1:10 using RNase-free water to obtain desired concentration per sample. Selection of the correct reference gene was based on comparison of stability and expression according to our experimental protocols and sample-set, assessed against a selection of reference candidates using a geNorm Kit (PrimerDesign Camberley, UK).

One-step RT-PCR was performed using the Precision®PLUS OneStep RT-qPCR Master Mix premixed with SYBRgreen (PrimerDesign, Camberley, UK) to a final reaction volume of 20 µl according to manufacturers' instructions. All reactions contained 2 µl of total RNA (at a total concentration of 25 ng), 10 µl of the Precision®PLUS OneStep RT-qPCR Master Mix premixed with SYBRgreen, 0.6 µl forward primer (at 6pmols), 0.6 µl reverse primer (at 6pmols), and 6.8 µl of RNase-free water. Each sample was assessed in triplicate, with no-template control

samples, containing RNase-free water in place of total RNA, being used for each run. All reactions were conducted according to the following reaction profile listed in Table 5.

Table 5. Details of RT-qPCR amplification conditions using SYBR Green PCR

	Step	Time	Temperature
	Reverse Transcription	10 minutes	55°C
	Enzyme Activation	2 minutes	95°C
CYCLING X 40	Denaturation	10 seconds	95°C
	Data Collection	60 seconds	60°C
	Melt Curve		50°C - 99°C

Table 6. Oligonucleotide primer and probe sequences using in quantitative real-time PCR.

Target	Primer sequence (5' - 3')	Probe sequence (5' - 3')	Source	C_t Threshold	Acc. No.
28S	F: GGCGAAGCCAGAGGAAACT R: GACGACCGATTTGCACGTC	P: (FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)	(Shini & Kaiser, 2009)	0.018	X59733
CXCLi2	F: GCCCTCCTCCTGGTTTCAG R: TGGCACCGCAGCTCATT	P: (FAM)-TCTTTACCAGCGTCCTACCTTGCGACA-(TAMRA)		0.0115	AJ009800
IFNγ	F: GTGAAGAAGGTGAAAGATATCATGGA R: GCTTTGCGCTGGATTCTCA	P: (FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)		0.018	Y07922
TGFβ$_4$	F: AGGATCTGCAGTGGAAGTGGAT R: CCCCGGGTTGTGTTGGT	P: (FAM)-ACCCAAAGGTTATATGGCCAACTTCTGCAT-(TAMRA)		0.006	M31160
IL-1β	F: GCTCTACATGTCGTGTGTGATGAG R: TGTCGATGTCCCGCATGA	P: (FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)		0.012	AJ245728
IL-4	F: AACATGCGTCAGCTCCTGAAT R: TCTGCTAGGAACTTCTCCATTGAA	P: (FAM)-AGCAGCACCTCCCTCAAGGCACC-(TAMRA)		0.008	AJ621249
IL-6	F: GCTCGCCGGCTTCGA R: GGTAGGTCTGAAAGGCGAACAG	P: (FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)		0.008	AJ250838
IL-10	F: CATGCTGCTGGGCCTGAA R: CGTCTCCTTGATCTGCTTGATG	P: (FAM)-CGACGATTCGGCGCTGTCACC-(TAMRA)		0.0115	AJ621614
IL-17A	F: CATGGGATTACAGGATCGATGA R: GCGGCACTGGGCATCA	P: (FAM)-ACAACCGCTTCCCCCGCTTGG-(TAMRA)	(Reid et al., 2016)	0.008	NM_204460.1
ACTB	F: AAGATCATTGCCCCACCTGA R: CCTGCTTGCTGATCCACCTGA		(John et al., 2017)	0.012	L08165.1
MUC2	F: ATGCGATGTTAACACAGGACTC R: GTGGAGCACAGCAGACTTTG		(Forder et al., 2012)	0.03	JX284122

Analysis of $2^{-\Delta\Delta}C_t$ RT-qPCR

For each expression triplicate per sample, an average Ct value was calculated based on threshold value used per gene of interest. All expression values for target genes were determined using $2^{-\Delta\Delta}C_t$ methodologies. Firstly, gene of interest (GOI) C_t was determined relative to that of the housekeeping 28S rRNA ($^{\Delta}C_t$). $^{\Delta}C_t$ values given for samples from *C. jejuni* infected birds were then normalised against those of uninfected control animals to give final readings as relative fold changes ($2^{-\Delta\Delta}C_t$). To determine statistical significance of variations in transcript expression between control and infected samples, pairwise comparisons of 40 - $^{\Delta}C_t$ was performed using Mann Whitney-U analysis, with statistical significance set at $p < 0.05$.

RESULTS

CAECAL COLONISATION

Caecal content was aseptically collected from all infected and control birds in both experimental trials 1 and 2 between 2 d.p.i – 28 d.p.i and 2 d.p.i – 21 d.p.i respectively (Figure 4). All birds within the non-infected control group were negative for *C. jejuni* caecal colonisation at all time-points in both experimental trials and will not be discussed in further detail. For all samples with detectable *C. jejuni* colonisation, counts of morphologically distinct bacterial colonies were taken at each post-mortem time-point. To account for any variation in sample weight, all values were subsequently corrected to 1 g total sample weight, with data presented as Colony Forming Units (CFU)/g. Caecal enumeration data sets for both Experiment 1 and Experiment 2 were non-normally distributed and, as such, will be discussed in regard to median and IQR values.

Caecal colonisation of *C. jejuni* was present from as early as 2 d.p.i in both experiment 1 (Figure 4a) and experiment 2 (Figure 4b) sample populations, found in 2/10 (20 %) and 4/6 (67 %) respectively. By 7 d.p.i, 9/11 (82 %) of the experiment 1 sample group had detectable *C. jejuni* within the caeca, with median bacterial loads of 6.92 Log_{10}CFU/g (IQR 4.72), with this being significantly higher than the *C. jejuni* burden of birds culled at 2 d.p.i ($p = 0.0128$). While colonisation load continued to rise between 7 and 14 d.p.i to 8.50 Log_{10}CFU/g (IQR 1.89), this was not found to be significantly higher than *C. jejuni* colonisation at 7 d.p.i ($p = 0.0671$). Maximal *C. jejuni* colonisation within experiment 1 was seen at 14 d.p.i and although bacterial

burden within the caeca remained high, ranging from 5.52 – 10.03 Log_{10}CFU/g between 21 to 28 d.p.i, there was no statistically significant increase between 14 d.p.i – 28 d.p.i (p = 0.6891).

Similar to experiment 1, *C. jejuni* burden at 7 d.p.i within experiment 2 was significantly higher than that seen for birds culled at 2 d.p.i within the sample experimental trial (p = 0.0216). This increase in *C. jejuni* burden between sampling time-points continued, with bacterial loads at 14 d.p.i being significantly higher than those seen at 7 d.p.i (p = 0.0006). As seen in experiment 1, 14 d.p.i was the time-point whereby maximal caecal *C. jejuni* colonisation was recorded, being 7.98 Log_{10}CFU/g (IQR 0.75). There was no significant increase in *C. jejuni* colonisation of the caeca between sample groups of 14 d.p.i and 21 d.p.i (p = 0.5338). Significance values for all pairwise treatment group comparisons referenced are provided in Table 7.

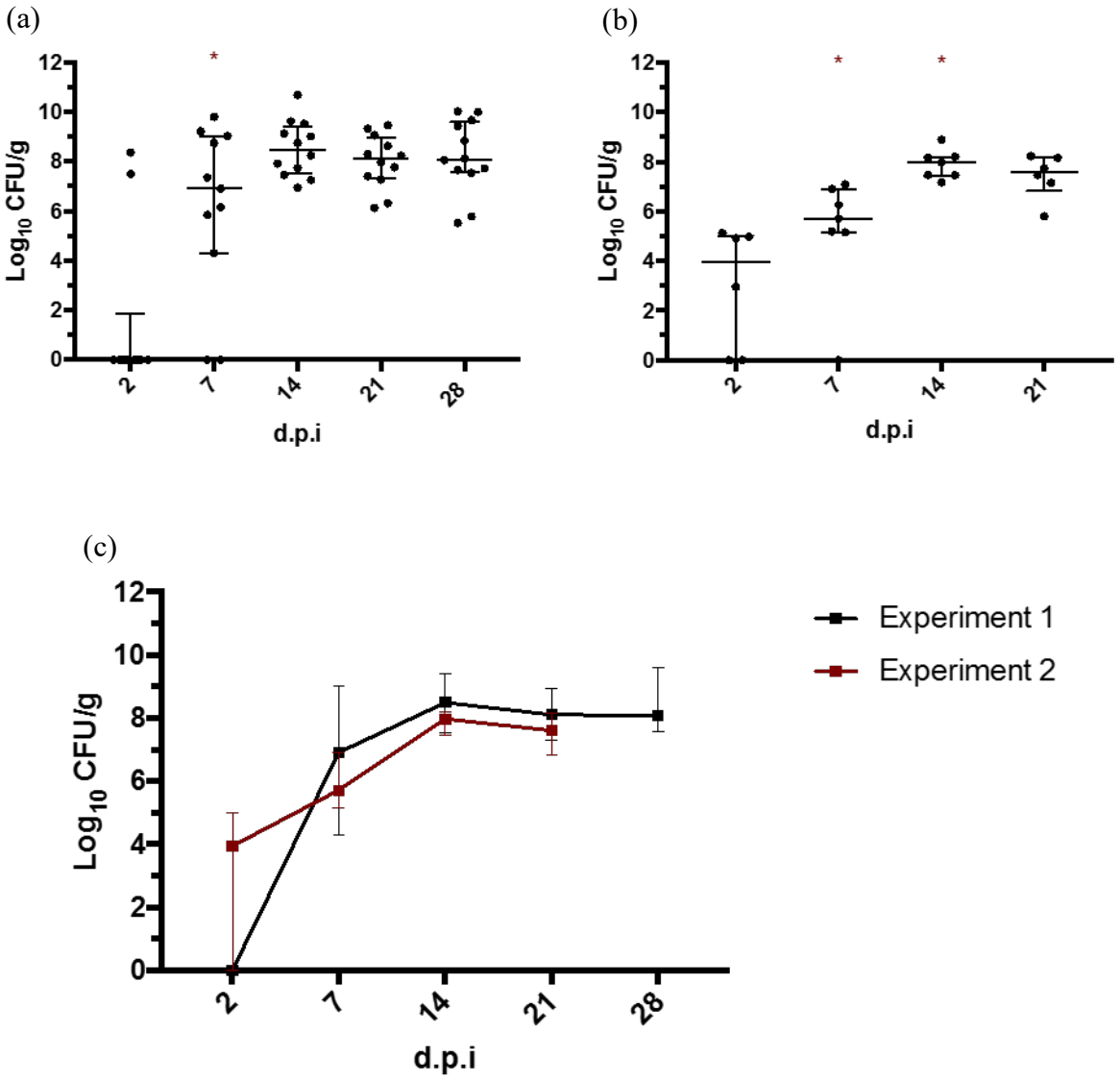


Figure 4. Levels of *C. jejuni* M1 in the caeca of *C. jejuni infected* broiler chickens under experimental conditions based on the protocols listed for experiments 1 (a) and 2 (b). Each symbol represents caecal *C. jejuni* load for an individual animal, with bars representing median values and their respective IQR. Comparable representation of caecal load between both experimental trials is shown in figure (c). Statistical significance was determined using Mann Whitney-U analysis, with '*' denoting time-points where *C. jejuni* load was statistically different from that of the immediately previous time-point ($p < 0.05$). Experient 1; 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12. Experiment 2; 2 d.p.i n=6, 7 & 14 d.p.i n=7, 21 d.p.i n=6.

Table 7. Statistical parameters and specific sampling time-point comparison significance values determined for Log_{10}CFU/g *C. jejuni* caecal colonisation

	Time point	Experiment 1						Experiment 2					
		C. jejuni Load (Log_{10}CFU/g)			Data normality	Group comparison	p value	*C. jejuni* Load (Log_{10}CFU/g)			Group comparison	p value
		Median	Quartiles					Median	Quartiles			
			Q1	Q2					Q1	Q2		
Caeca	2	0	0	1.88	0.021	7	0.0128	3.94	0	5.01	7	0.0216
						14	0.0002				14	0.0012
						21	0.0006				21	0.0022
						28	0.0002				N/A	N/A
	7	6.92	4.32	9.04	0.29	14	0.0671	5.71	5.15	6.9	14	0.0006
						21	0.2292				21	0.0082
						28	0.1301				N/A	N/A
	14	8.5	7.54	9.43	0.79	21	0.3777	7.98	7.45	8.2	21	0.5338
						28	0.8428				N/A	N/A
	21	8.1	7.31	8.96	0.747	28	0.6707	7.6	6.82	8.18	N/A	N/A
	28	8.09	7.58	9.61	0.6639	N/A	N/A					

ILEAL COLONISATION

Ileal content was aseptically collected from all infected and non-infected control birds in both experimental trials 1 and 2 between 2 d.p.i – 28 d.p.i and 2 d.p.i – 21 d.p.i respectively (Figure 5a). In accordance with the information attained for caecal colonisation, all birds within the non-infected control groups of both experiment 1 and experiment 2 showed no detectable *C. jejuni* colonisation within the ileum and will not be discussed in further detail. Processing of ileal samples was conducted in the same manner as for caecal samples with presentation of data as Log_{10}CFU/g. Ileal enumeration data sets for both experiment 1 and experiment 2 were non-normally distributed and as such, will be discussed in regard to median and IQR values.

C. jejuni colonisation within the ileum was notably less prominent compared to that of the caecum. In both experiment 1 and experiment 2, detection of *C. jejuni* was first observed at 7 d.p.i in 1/11 (9 %) and 2/7 (29 %) samples respectively. While colonisation of the ileum within experiment 1 showed highest frequency in samples collected 14 d.p.i (7/12 [58 %]), persistence of *C. jejuni* colonisation within the ileum was markedly reduced compared to that of the caecum, with colonisation detected in only 1/12 (8 %) and 3/12 (25 %) samples 21 and 28 d.p.i respectively (Figure 5b). In addition to lesser colonisation frequency compared to the caecum, *C. jejuni* load within the ileum was considerably lower, with a range of 3.57 – 7.74 Log_{10}CFU/g across all sampled time-points within experiment 1.

As with experiment 1, ileal colonisation within experiment 2 was considerably less frequent compared to that of caecal samples obtained from the same experimental birds. Maximal ileal colonisation within experiment 2 was observed at 21 d.p.i with 3/6 (50 %) samples showing *C. jejuni* colonisation at a load ranging from 4.45 – 5.16 Log_{10}CFU/g. Frequency of *C. jejuni* detection within sampled ileal content at each time point of experiment 1 and experiment 2 is shown in Figure 5c.

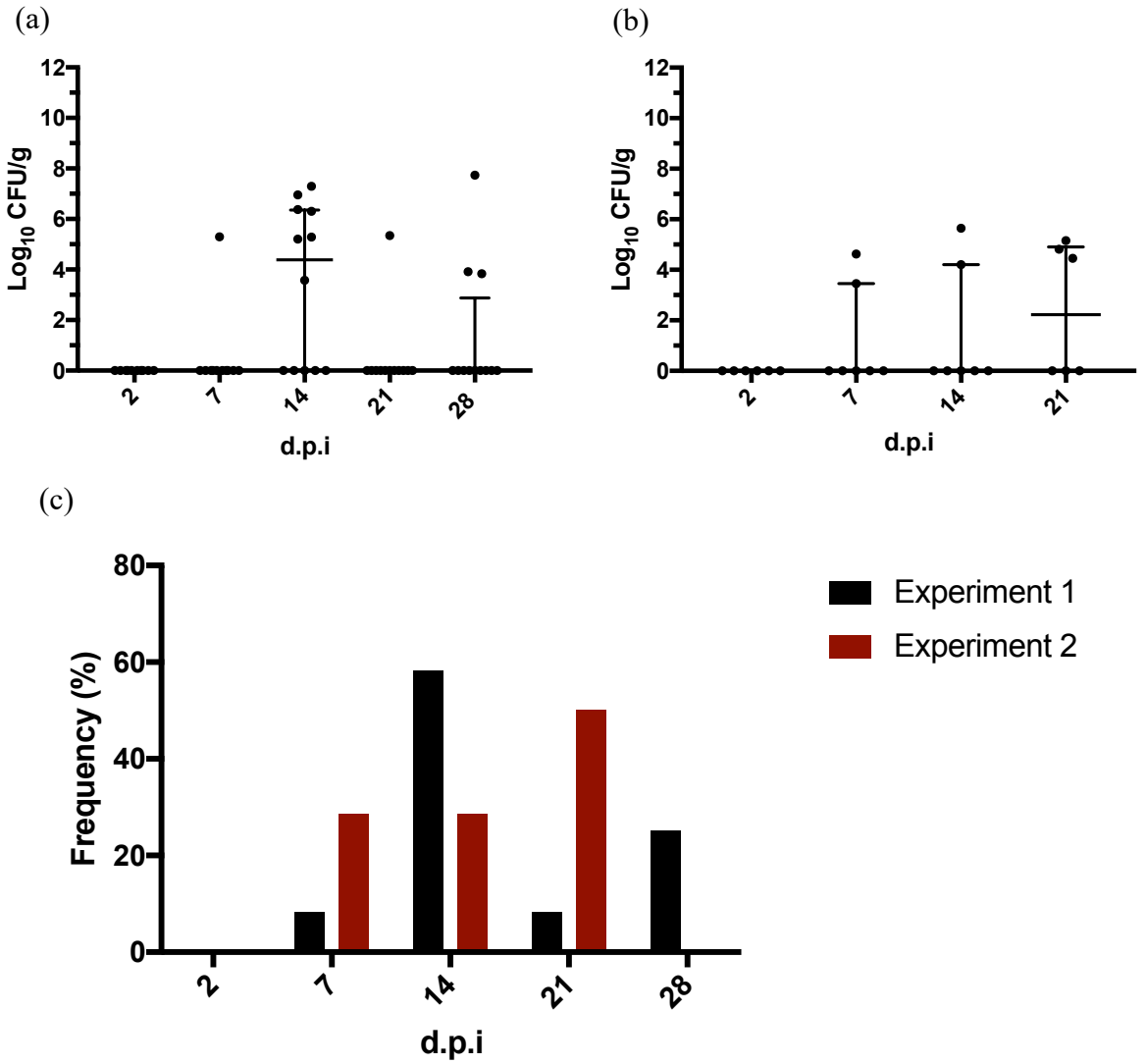


Figure 5. Levels of *C. jejuni* M1 in the ileum of broiler chickens under experimental conditions based on the protocols listed for experiments 1 (a) and 2 (b). Each symbol represents caecal *C. jejuni* load for an individual animal, with bars representing median values and their respective IQR. Percentage frequency of ileal *C. jejuni* colonisation out of total sample population per time-point for both experimental protocols is provided in figure (c). Experient 1; 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12. Experiment 2; 2 d.p.i n=6, 7 & 14 d.p.i n=7, 21 d.p.i n=6.

EXTRA-INTESTINAL SPREAD OF *C. JEJUNI*

Tissue samples from both the spleen and liver were collected from all birds at post-mortem in both experimental trials 1 and 2 between 2 d.p.i – 28 d.p.i and 2 d.p.i – 21 d.p.i respectively to assess the ability of *C. jejuni* M1 to establish beyond the GIT. All birds in the non-infected control groups of both experiment 1 and experiment 2 showed no detectable *C. jejuni* colonisation of either tissue and will not be discussed in any further detail.

C. jejuni was identified as early as 2 d.p.i within the splenic tissue of sampled birds from experiment 1 (Figure 6). While detection remained at a relatively low frequency, detection of *C. jejuni* within at least one spleen sample occurred at all sampling time-points within this experimental trial. Maximal colonisation frequencies occurred later after infection, with 2/12 (16 %) spleen samples at both 21 d.p.i and 28 d.p.i detected as *C. jejuni* positive. Detection of *C. jejuni* within the spleen was detected slightly later, at 7 d.p.i within experiment 2. Frequency of *C. jejuni* detection within the spleen was highest at 21 d.p.i with 2/6 (33 %) samples positive for the bacteria.

C. jejuni invasion of the hepatic tissues was observed later than that seen for splenic tissues in both experiment 1 and experiment 2 (Figure 5). First detection of *C. jejuni* was observed 7 d.p.i in 1/11 (9 %) birds sampled within experiment 1. As with invasion of splenic tissues, *C. jejuni* was detected more frequently in samples taken at later time-points, with 4/12 (33 %) samples positive for *C. jejuni* at 28 d.p.i within experiment 1. Detection of *C. jejuni* within the liver tissue was first observed 14 d.p.i within experiment 2 and showed maximal incidence frequency as 21 d.p.i with 3/6 (50 %) of samples positive.

	Bird ID	Spleen															
		D	E	Bird ID	D	E	Bird ID	D	E	Bird ID	D	E	Bird ID	D	E		
		2			7			14			21			28			
Exp. 1	1937			1935			1936			1934			1941				
	1942			1940			1938			1944			1953				
	1946			1947			1939			1945			1955				
	1950			1952			1943			1948			1956				
	1951			1959			1949			1958			1966				
	1968			1962			1954			1963			1967				
	1970			1965			1957			1964			1975				
	1988			1971			1969			1972			1979				
	1990			1973			1974			1977			1981				
	1991			1984			1976			1980			1985				
				1989			1978			1982			1987				
							1983			1986			1992				
Exp. 2	1			7			14			21							
	2			8			15			22							
	3			9			16			23							
	4			10			17			24							
	5			11			18			25							
	6			12			19			26							
				13			20										

Figure 6. Detection of *C. jejuni* M1 within spleen tissue of broiler chickens under experimental conditions based on the protocols listed for experiment 1 and experiment 2. Red squares depict *C. jejuni* detection within a single sample, whereby 'D' indicates results are from direct plating of tissue homogenate and 'E' depicts results are from enriched samples. Experient 1; 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12. Experiment 2; 2 d.p.i n=6, 7 & 14 d.p.i n=7, 21 d.p.i n=6.

	Liver																	
	Bird ID	D	E	Bird ID	D	E	Bird ID	D	E	Bird ID	D	E	Bird ID	D	E
		2			7			14			21			28	
Exp. 1	1937			1935			1936			1934			1941		
	1942			1940			1938			1944			1953		
	1946			1947			1939			1945			1955		
	1950			1952			1943			1948			1956		
	1951			1959			1949			1958			1966		
	1968			1962			1954			1963			1967		
	1970			1965			1957			1964			1975		
	1988			1971			1969			1972			1979		
	1990			1973			1974			1977			1981		
	1991			1984			1976			1980			1985		
				1989			1978			1982			1987		
							1983			1986			1992		
Exp. 2	1			7			14			21					
	2			8			15			22					
	3			9			16			23					
	4			10			17			24					
	5			11			18			25					
	6			12			19			26					
				13			20								

Figure 7. Detection of *C. jejuni* M1 within liver tissue of broiler chickens under experimental conditions based on the protocols listed for experiment 1 and experiment 2. Red squares depict *C. jejuni* detection within a single sample, whereby 'D' indicates results are from direct plating of tissue homogenate and 'E' depicts results are from enriched samples. Experient 1; 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12. Experiment 2; 2 d.p.i n=6, 7 & 14 d.p.i n=7, 21 d.p.i n=6.

To assess whether high caecal *C. jejuni* colonisation was a predictor for systemic bacterial spread, splenic and hepatic tissue *C. jejuni* positivity was related back to caecal *C. jejuni* load, with this being shown in Figure 8. There appears to be no visible association between extra-intestinal spread of *C. jejuni* and caecal *C. jejuni* burden in either experiment 1 or experiment 2.

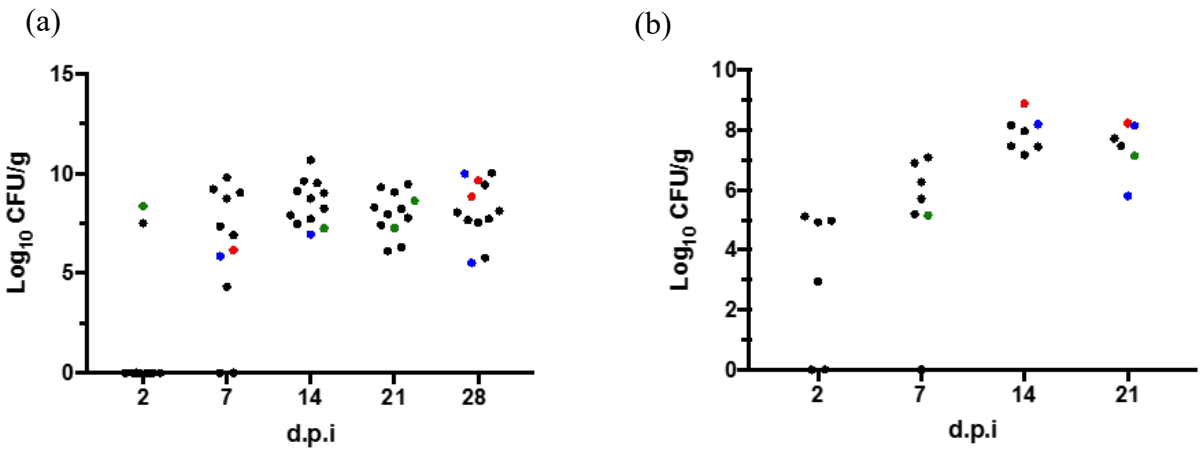


Figure 8. Extra-intestinal detection of *C. jejuni* mapped against *C. jejuni* caecal load for broiler chickens under experimental conditions based on experimental protocols 1 (a) & 2(b). Each symbol represents results from an individual animal with caecal load given as Log_{10}CFU/g of caecal content. Green shapes indicate birds with *C. jejuni* detected in splenic tissue, blue shapes show detection in liver tissue and red shapes represent animals with *C. jejuni* detected in both splenic and liver tissues. Experient 1; 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12. Experiment 2; 2 d.p.i n=6, 7 & 14 d.p.i n=7, 21 d.p.i n=6.

ELISA

Serum IgY and IgM

Samples of 2 ml whole blood were collected from each bird in both infected and non-infected groups of experiment 1 and experiment 2 via cardiac puncture at post-mortem. Serum samples were prepared and measured for specific IgY and IgM against *C. jejuni*. Following antigen preparation protocols, whole cell *C. jejuni* lysate antigen was prepared at a concentration of 257.35 µg/ml following interpolation from the plotted standard curve provided in Figure 9.

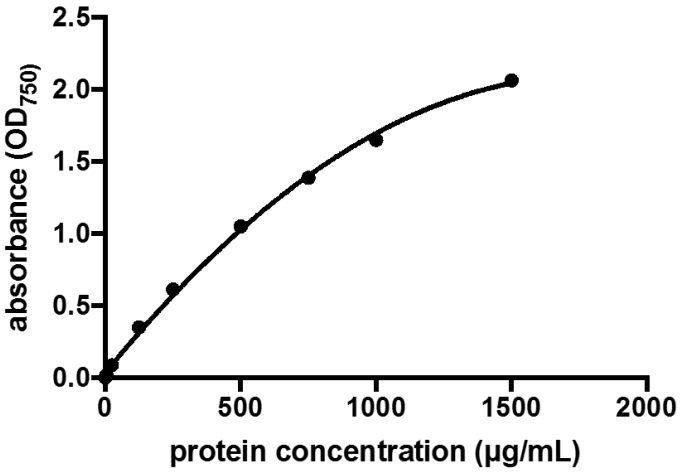


Figure 9. Standard curve representing OD_{750} against protein concentration for interpolation of soluble antigen concentrations.

Within both experiments, the level of serum IgY was similar at 2 d.p.i between *C. jejuni* infected and non-infected birds (Experiment 1 $p = 0.8639$; Experiment 2 $p = 0.9372$) (Figure 10). Within experiment 1, IgY levels for non-infected control birds remained relatively low across all time-points, with an OD_{405} absorbance range of 0.24 – 0.82. From 7 d.p.i onward, *C. jejuni* infected birds showed significantly higher serum IgY compared to non-infected birds sampled at the same time-point ($p < 0.05$). Serum IgY was highest within this experiment at 21 d.p.i, with median OD_{405} of 1.60 (IQR 0.55). A similar IgY response was observed for experiment 2, with peak OD_{405} of 0.90 (IQR 0.64) at 21 d.p.i and persistently low serum IgY of non-infected control birds ranging from 0.017 – 0.23. Details of statistical parameters and pairwise group comparison significance values are provided in Table 8.

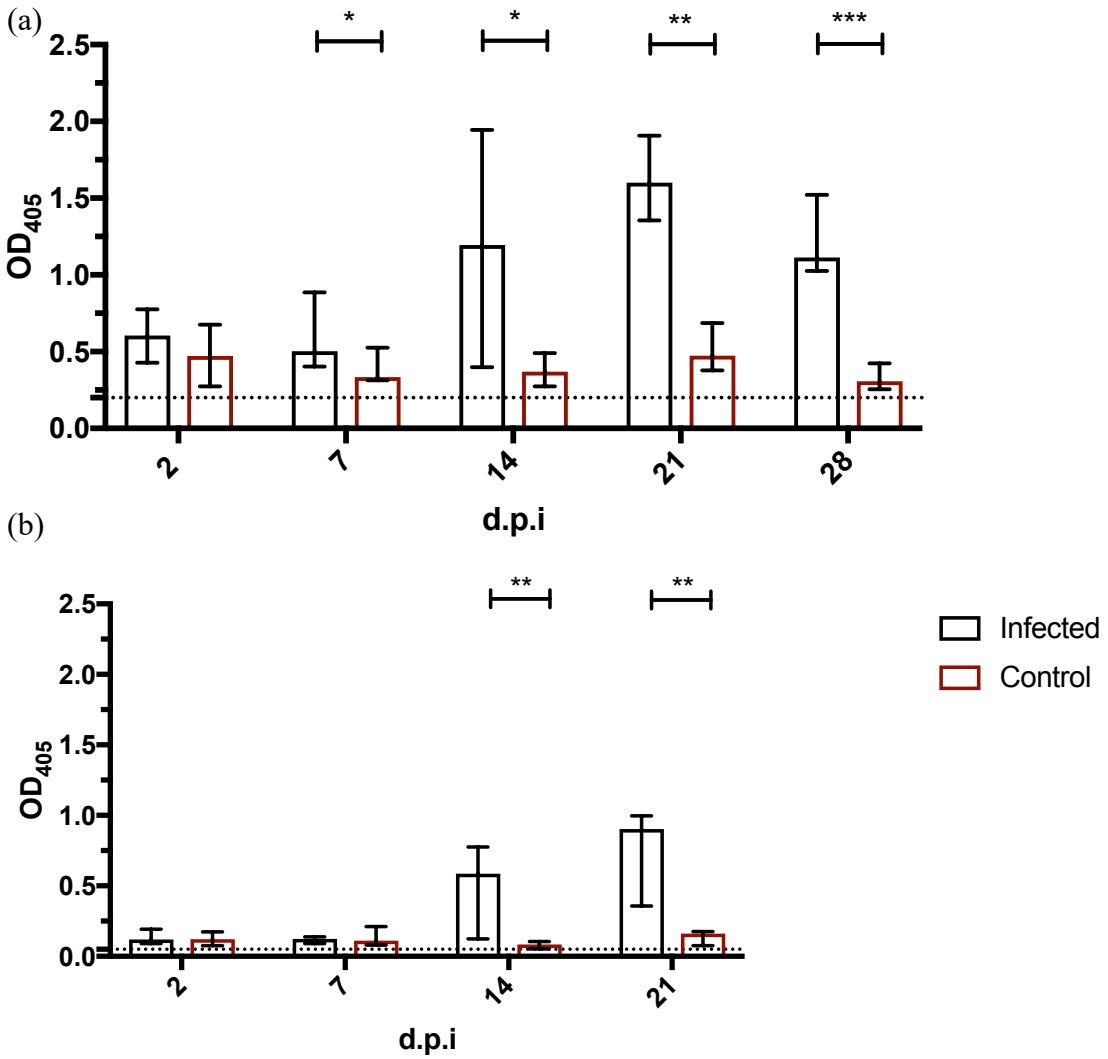


Figure 10. Normalised serum IgY response according to *C. jejuni* M1 infection status at samples time-points post-challenge according to experimental protocols for experiment 1 (a) and experiment 2 (b). Statistical analysis is based on median values with associated IQR. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p < 0.05, **p < 0.01, ***p < 0.001. The dotted line of both plots indicates the average absorbance for plate negative control samples. Experient 1; 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12. Experiment 2; 2 d.p.i n=6, 7 & 14 d.p.i n=7, 21 d.p.i n=6.

As with serum IgY, serum IgM levels remained similar between *C. jejuni* infected and non-infected birds early after infection (2 d.p.i) ($p < 0.05$) (Figure 11).However, by 14 d.p.i in experiment 1, serum IgM in infected birds was significantly higher than that of non-infected control birds ($p = 0.0001$). Peak serum IgM within infected birds was observed at 21 d.p.i, with median levels of OD_{405} 2.23 (IQR 1.28), with this being significantly above that of non-infected controls at the same-time point ($p = 0.0008$). Interestingly, although IgM was still increased within infected chickens compared to non-infected at 28 d.p.i ($p = 0.0069$), levels were lower than that of infected birds at 21 d.p.h.

As with experiment 1, increases in serum IgM levels of *C. jejuni* infected birds were first identified at 14 d.p.i (p = 0.0043). This continued at 21 d.p.i, whereby median levels reached 1.01 (IQR 0.95) within *C. jejuni* infected birds, considerably higher than that of non-infected birds' samples at that time (p = 0.014). Details of statistical parameters and pairwise group comparison significance values are provided in Table 8.

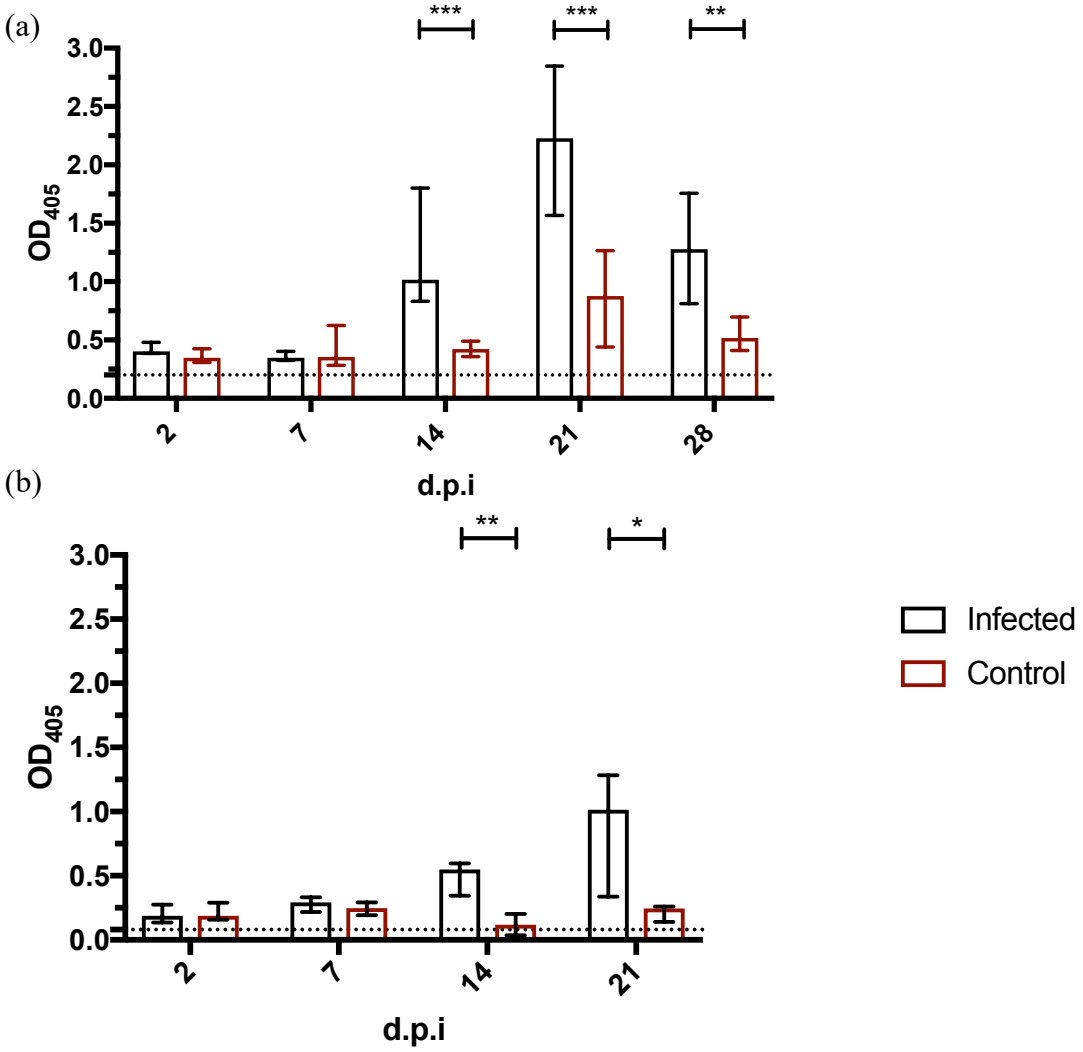


Figure 11. Normalised serum IgM response according to *C. jejuni* M1 infection status at samples time-points post-challenge according to experimental protocols for experiment 1 (a) and experiment 2 (b). Statistical analysis is based on median values with associated IQR. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p < 0.05, **p < 0.01, ***p < 0.001. The dotted line of both plots indicates the average absorbance for plate negative control samples. Infected 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12; Control 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12.

Table 8. Statistical parameters and pairwise comparison significance values determined for *C. jejuni* specific serum IgY and IgM (OD_{405}). All *p* values are provided on infected against non-infected control comparison at each given time point.

Immunoglobulin	d.p.i	Challenge status	Experiment 1				Experiment 2			
			OD_{405}			*p*-value	OD_{405}			*p*-value
			Median	Quartiles			Median	Quartiles		
				Q1	Q3			Q1	Q3	
IgY	2	Infected	0.6049	0.4283	0.7759	0.8639	0.1205	0.08973	0.1934	0.9372
		Control	0.4714	0.274	0.6769		0.1223	0.07599	0.1745	
	7	Infected	0.5029	0.4027	0.8856	0.0312	0.1234	0.0901	0.1391	0.9015
		Control	0.3336	0.3143	0.5262		0.1124	0.0811	0.2121	
	14	Infected	1.195	0.3984	1.945	0.0668	0.5856	0.1241	0.7753	0.0022
		Control	0.3708	0.274	0.4915		0.08408	0.05389	0.1044	
	21	Infected	1.601	1.356	1.906	0.0013	0.9033	0.357	0.9965	0.0012
		Control	0.4739	0.3777	0.6874		0.1623	0.07665	0.1751	
	28	Infected	1.113	1.026	1.522	0.0013				
		Control	0.3065	0.2545	0.4245					
IgM	2	Infected	0.4032	0.3847	0.4796	0.0663	0.1886	0.136	0.2759	0.9372
		Control	0.3483	0.308	0.4263		0.1892	0.1581	0.2906	
	7	Infected	0.3471	0.3264	0.4016	0.9578	0.2922	0.2182	0.3329	0.3829
		Control	0.3555	0.2825	0.6236		0.2481	0.194	0.2931	
	14	Infected	1.017	0.8307	1.803	0.0001	0.5493	0.3448	0.5962	0.0043
		Control	0.4221	0.3567	0.4894		0.1172	0.03683	0.2034	
	21	Infected	2.228	1.567	2.845	0.0008	1.014	0.3379	1.283	0.014
		Control	0.8756	0.44	1.265		0.2463	0.14	0.2614	
	28	Infected	1.278	0.8114	1.758	0.0069				
		Control	0.5186	0.41	0.6965					

Secretory IgA

Sections of ileal tissue were collected from all *C. jejuni* infected and non-infected control birds of Experiment 1 at post-mortem to determine the levels of total secretory IgA (Figure 12). Total secretory IgA within non-infected control chickens showed considerably higher variation at early time-points compared to that of *C. jejuni* infected birds, with concentrations ranging from 1.13 – 54.49 ng/ml and 0.39 – 21.71 ng/ml respectively at 2 d.p.i. Total secretory IgA was similar in *C. jejuni* infected and uninfected birds at 7 and 14 d.p.i (p = 0.5908; p = > 0.9999 respectively). While total IgA appeared to show gradual decline within uninfected control birds from 14 d.p.i, the opposite was true for *C. jejuni* infected birds, with significantly higher IgA at both 21 d.p.i (p = 0.0240) and 28 d.p.i (p = 0.004). Details of statistical parameters and pairwise group comparison significance values are provided in Table 9.

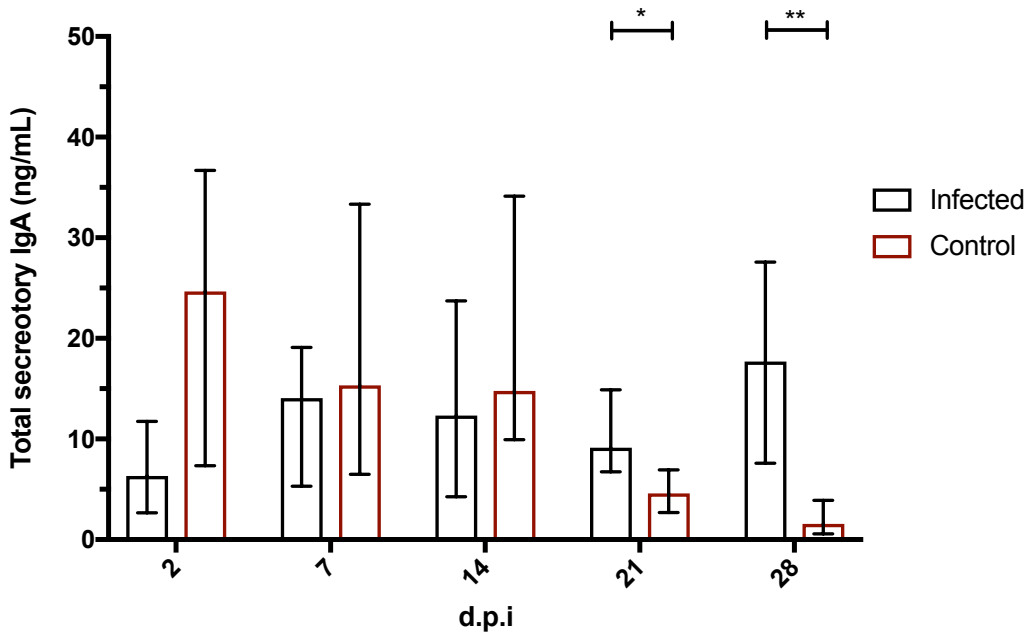


Figure 12. Total secretory IgA response within ileal tissue according to *C. jejuni* M1 infection status at sampled time-points post-challenge according to experimental protocols for experiment 1 (a) and experiment 2 (b). Statistical analysis is based on median values with associated IQR. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p < 0.05, **p < 0.01. Experient 1; 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12. Experiment 2; 2 d.p.i n=6, 7 & 14 d.p.i n=7, 21 d.p.i n=6.

Table 9. Statistical parameters and pairwise comparison significance values determined for total secretory IgA. All *p* values are provided on infected against non-infected control comparison at each given time point.

Immunoglobulin	d.p.i	Challenge status	IgA (ng/ml)			p-value
			Median	Quartiles		
				Q1	Q3	
IgA	2	Infected	6.306	2.662	11.73	0.0663
		Control	24.67	7.337	36.7	
	7	Infected	14.05	5.292	19.08	0.5908
		Control	15.32	6.493	33.33	
	14	Infected	17.69	7.599	27.57	>0.9999
		Control	14.77	9.936	34.12	
	21	Infected	9.116	6.736	14.88	0.024
		Control	4.573	2.676	6.936	
	28	Infected	17.69	7.599	27.57	0.004
		Control	1.545	0.5684	3.88	

CYTOKINE EXPRESSION BY RELATIVE $2^{-\Delta\Delta CT}$ RT-qPCR

2 d.p.i

To assess the presence of inflammatory response to *Campylobacter* challenge, specific cytokine, chemokine and glycoprotein expression within the caecal and caecal tonsil tissue of all birds from experiment 1 was quantified relative to non-infected control birds (Figure 13, Table 10, Table 11). *Campylobacter jejuni* challenge was determined to elicit time-dependent alterations in immune response and regulation over the duration of the experiment. Here expression levels of the cytokine gene transcripts IL-1β, IL-4, IL-6, IL-10, IL-17A and $TGF\beta_4$ alongside the chemokine CXCLi2 were targeted as inducers or mediators of major immune pathways. While IL-1β, IL-6, IL-17A, and CXCLi2 are all strongly associated with a pro-inflammatory Th17 pathway response, IL-4 is associated with a Th2 response and IL-10 an anti-inflammatory regulatory T cell (Treg response) (Connerton et al., 2018; Reid et al., 2016). $TGF\beta_4$ is often implicated in a Th17 role however strongly influences multiple Treg functions. At 2 d.p.i, an innate upregulation of IL-1β, IL-6 and CXCLi2 was seen within the caecal tissue ($p < 0.01$) (Figure 13). All are known inducers of pro-inflammatory immune responses and potent initiators of further Th17 pathways. In contract, IL-1β and IL-17A were downregulated within the caecal tonsil by 0.27 (IQR 0.27) and 0.27 (IQR 0.19) -fold respectively ($p < 0.05$) at the same time-point. Infection status did not influence the gene expression of any other gene transcript in either tissue type ($p > 0.05$). It may be of importance to note that at the 2 d.p.i time-point,

only 2/10 samples chickens orally infected with *C. jejuni* were detected as being *C. jejuni* positive.

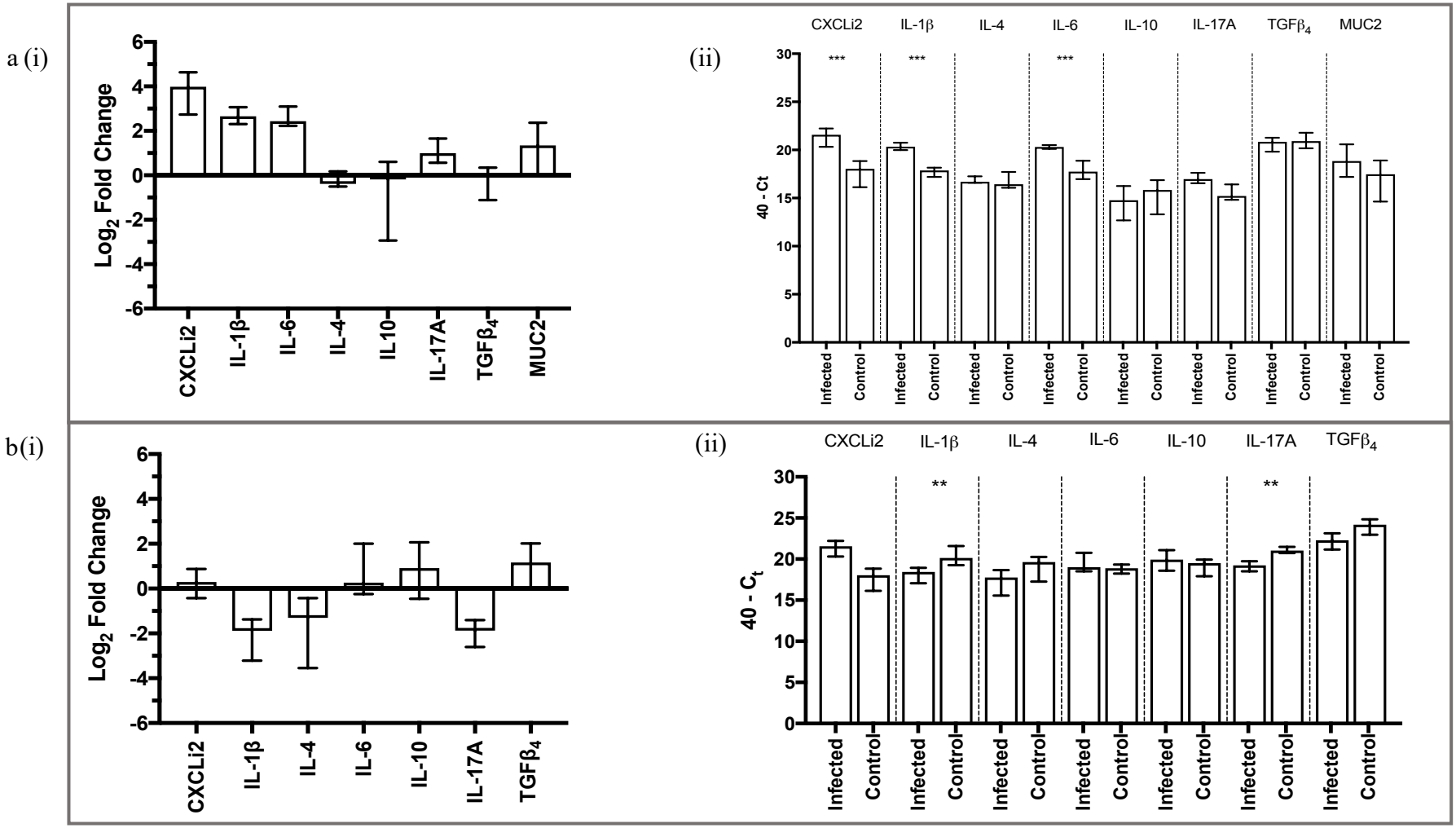


Figure 13. Relative expression (i) and 40 - $^{\Delta}C_t$ (ii) of assessed cytokine, chemokine and AMP transcripts within caecal tissue (a) and caecal tonsil tissue (b) of experimental chickens according to *C. jejuni* challenge status at 2 d.p.i (23 d.p.h). Error bars represent IQR of the median value and statistical significance has been assessed according to 40 - $^{\Delta}C_t$. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as **p < 0.01, ***p < 0.001. Infected 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12; control 2, 7, 14, 21 & 28 d.p.i n=6.

7 d.p.i

By 7 d.p.i, most cytokines associated with the Th17 response were upregulated within the caecal tissue in accordance with the increase in caecal colonisation seen within the bacteriological results for this time-point. ($p < 0.05$) (Figure 14). In conjunction with the pro-inflammatory cytokines mentioned previously, $TGF\beta_4$ showed significant upregulation by 7 d.p.i ($p < 0.01$) of 4.57 (IQR 3.60) fold. With primary influence in initiation of the Treg pathways, this was accompanied by an increase of 3.57 (IQR 4.78) fold in IL-10 within the caeca, although such change was not of statistical significance ($p < 0.05$). An increase in IL-4 transcripts provides evidence pertaining to the activation of the Th2 pathways by this time-point and may have been stimulated by the early innate production of IL-6 within the caecal tissues ($p < 0.05$) (Reid et al., 2016). Unseen at 2 d.p.i, transcription of the gel-forming mucin MUC2 was significantly downregulated in *C. jejuni* infected chickens compared to non-infected controls ($p < 0.001$). While mucins form a major component of the physical mucus barrier protecting the avian intestinal tract they have been denoted as an environmental trigger for the production of several pathogenicity and colonisation factors from *C. jejuni* found within the caecal intestinal mucus (Tu et al., 2008).

Caecal tonsil tissues had markedly different cytokine expression profiles relative to non-infected control birds compared to that at 2 d.p.i. A continued up-regulation of IL-1β by 3.46 (IQR 2.40) -fold supplemented by upregulation of IL-6 and IL-17A perpetuates the pro-inflammatory response seen within the caecal tissue ($p < 0.05$) (Figure 14). As seen within the caecal tissue, increases in $TGF\beta_4$ expression were evident by 7 d.p.i within the caecal tonsil ($p < 0.01$). This 2.84 (IQR 1.92) fold $TGF\beta_4$ up-regulation may play an instrumental role in activation of the same Treg pathway associated with the accompanying IL-10 upregulation ($p < 0.001$).

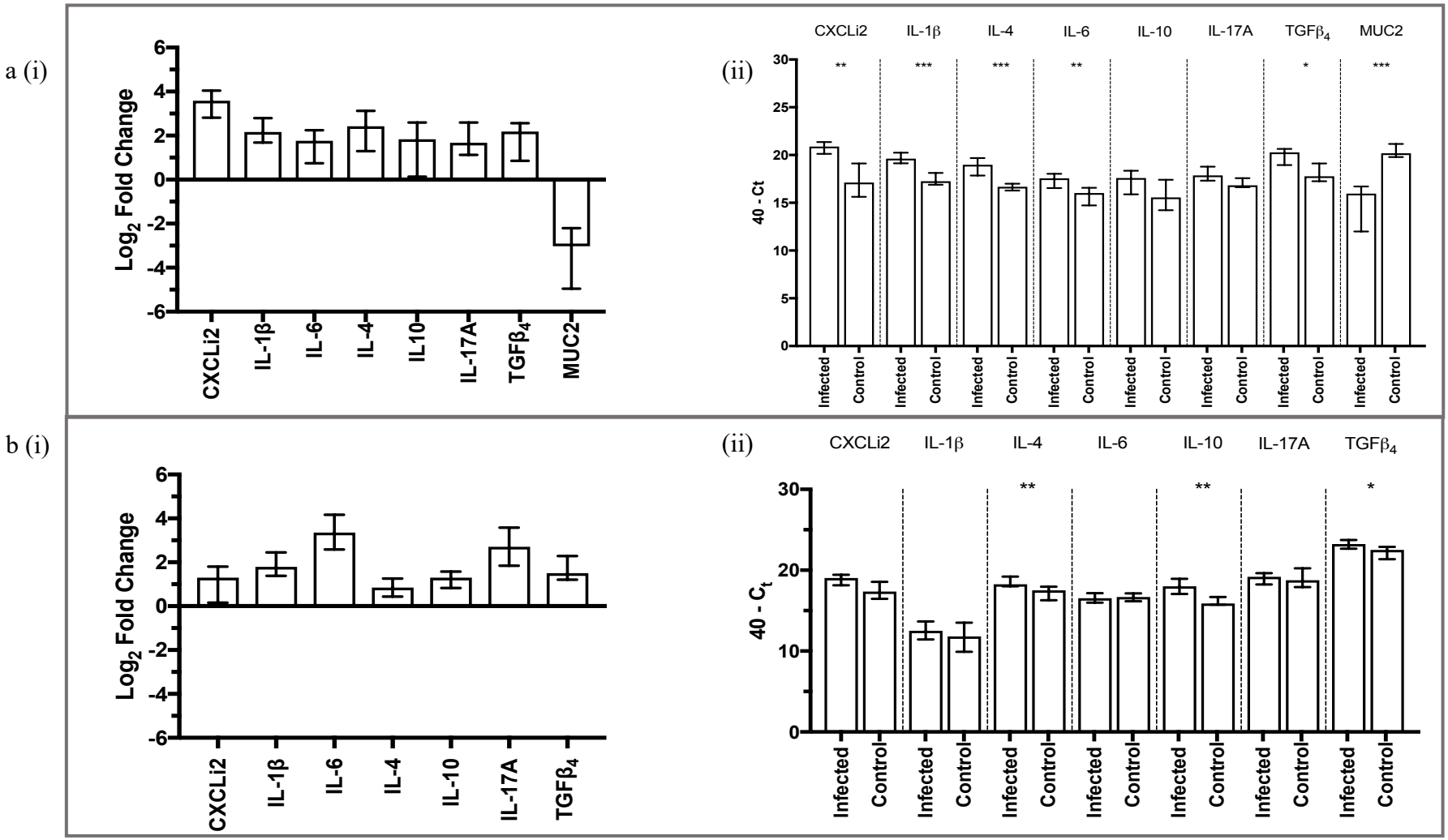


Figure 14. Relative expression (i) and 40 - $^{\Delta}C_t$ (ii) of assessed cytokine, chemokine and AMP transcripts within caecal tissue (a) and caecal tonsil tissue (b) of experimental chickens according to *C. jejuni* challenge status at 7 d.p.i (23 d.p.h). Error bars represent IQR of the median value and statistical significance has been assessed according to 40 - $^{\Delta}C_t$. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Infected 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12; control 2, 7, 14, 21 & 28 d.p.i n=6.

14 d.p.i

By 14 d.p.i, there appears to be a marked shift in the characteristics of immune response to *C. jejuni* challenge within both the caecal and caecal tonsil tissues. The upregulation of the innate pro-inflammatory cytokines IL-1β and IL-6 has diminished but may have in turn stimulated an increase in IL-17A expression ($p < 0.01$). Caecal TGFβ4 upregulation is evident by 2.16 (IQR 2.82)-fold, continuing from that seen at 7 d.p.i. ($p < 0.05$). Expression of IL-4 continues to be elevated within the caecal tissues ($p < 0.05$) alongside the caecal tonsil ($p < 0.01$) at 14 d.p.i. The anti-inflammatory nature of IL-10 foresees its upregulation at 14 d.p.i and is likely positively correlated with both the array of pro-inflammatory transcript expression seen at 7 d.p.i and continued $TGF\beta_4$ upregulation.

21 d.p.i & 28 d.p.i

During the later time-points post-infection, relative expression of all analyzed transcripts remained similar to those of non-infected control birds. Within the caeca, both IL-6 and CXCLi2 showed further up-regulation by 2.68 (IQR 3.76) fold and 5.25 (IQR 2.17) fold respectively, however this pro-inflammatory response was likely countered by parallel increases in IL-10 and $TGF\beta_4$ (Figure 16, Figure 17). This increase in regulatory pathways continues into 28 d.p.i within the caeca whereby IL-10 shows 12.83 (IQR 11.30) fold upregulation (Figure 17). This regulatory response is largely mirrored within the caecal tonsil across both 21 and 28 d.p.i, with upregulation of IL-10 (Figure 16, Figure 17). However an additional Th2 response continues from 14 d.p.i across both 21 and 28 d.p.i, with further IL-4 upregulation by 2.15 (IQR 1.56) fold and 3.59 (IQR 3.35)-fold respectively.

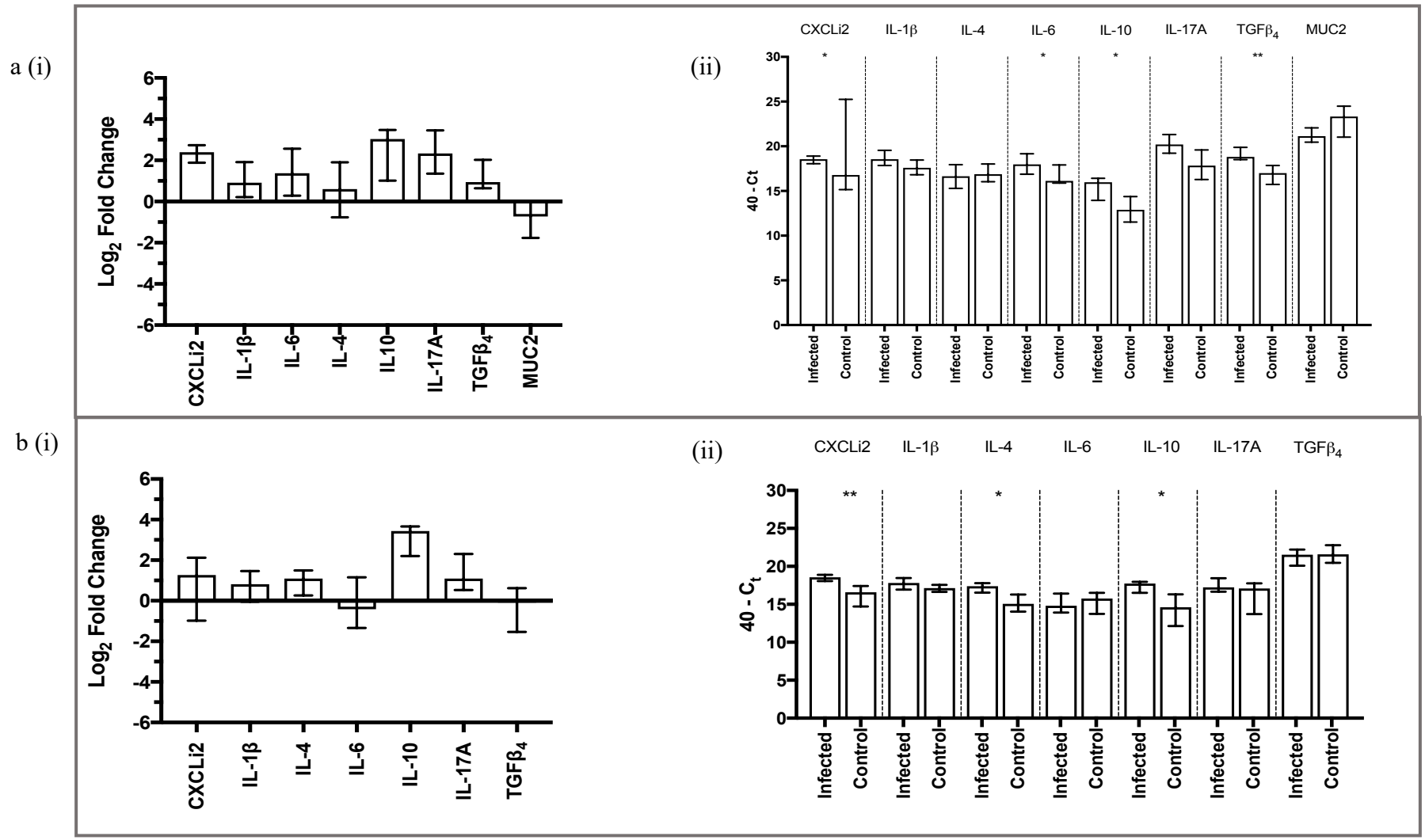


Figure 15. Relative expression (i) and 40 - $^{\Delta}C_t$ (ii) of assessed cytokine, chemokine and AMP transcripts within caecal tissue (a) and caecal tonsil tissue (b) of experimental chickens according to *C. jejuni* challenge status at 14 d.p.i (23 d.p.h). Error bars represent IQR of the median value and statistical significance has been assessed according to 40 - $^{\Delta}C_t$. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as * p < 0.05, **p < 0.01. Infected 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12; control 2, 7, 14, 21 & 28 d.p.i n=6.

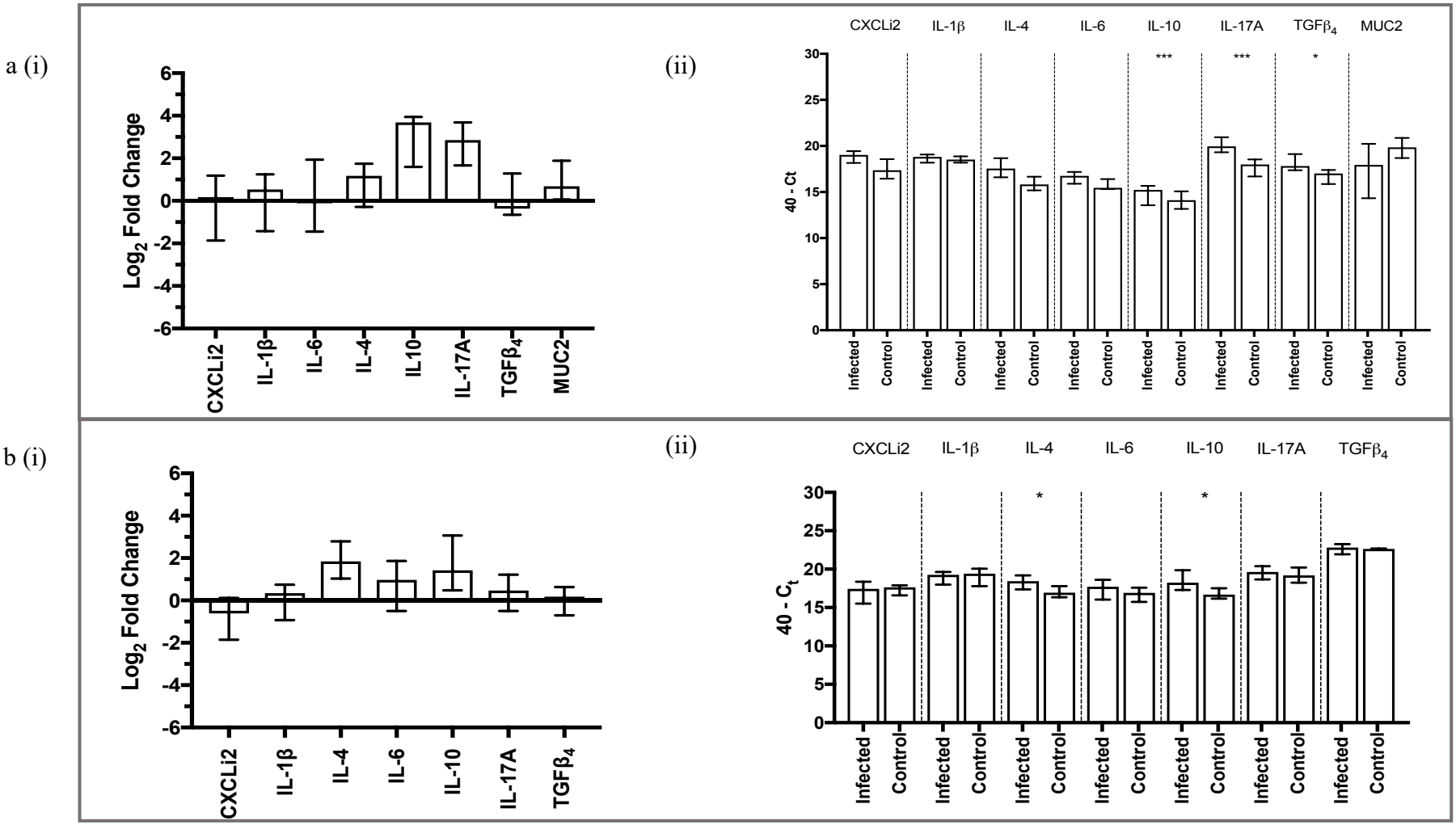


Figure 16. Relative expression (i) and 40 - $^{\Delta}C_t$ (ii) of assessed cytokine, chemokine and AMP transcripts within caecal tissue (a) and caecal tonsil tissue (b) of experimental chickens according to *C. jejuni* challenge status at 21 d.p.i (23 d.p.h). Error bars represent IQR of the median value and statistical significance has been assessed according to 40 - $^{\Delta}C_t$. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as * $p < 0.05$, ***$p < 0.001$. Infected 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12; control 2, 7, 14, 21 & 28 d.p.i n=6.

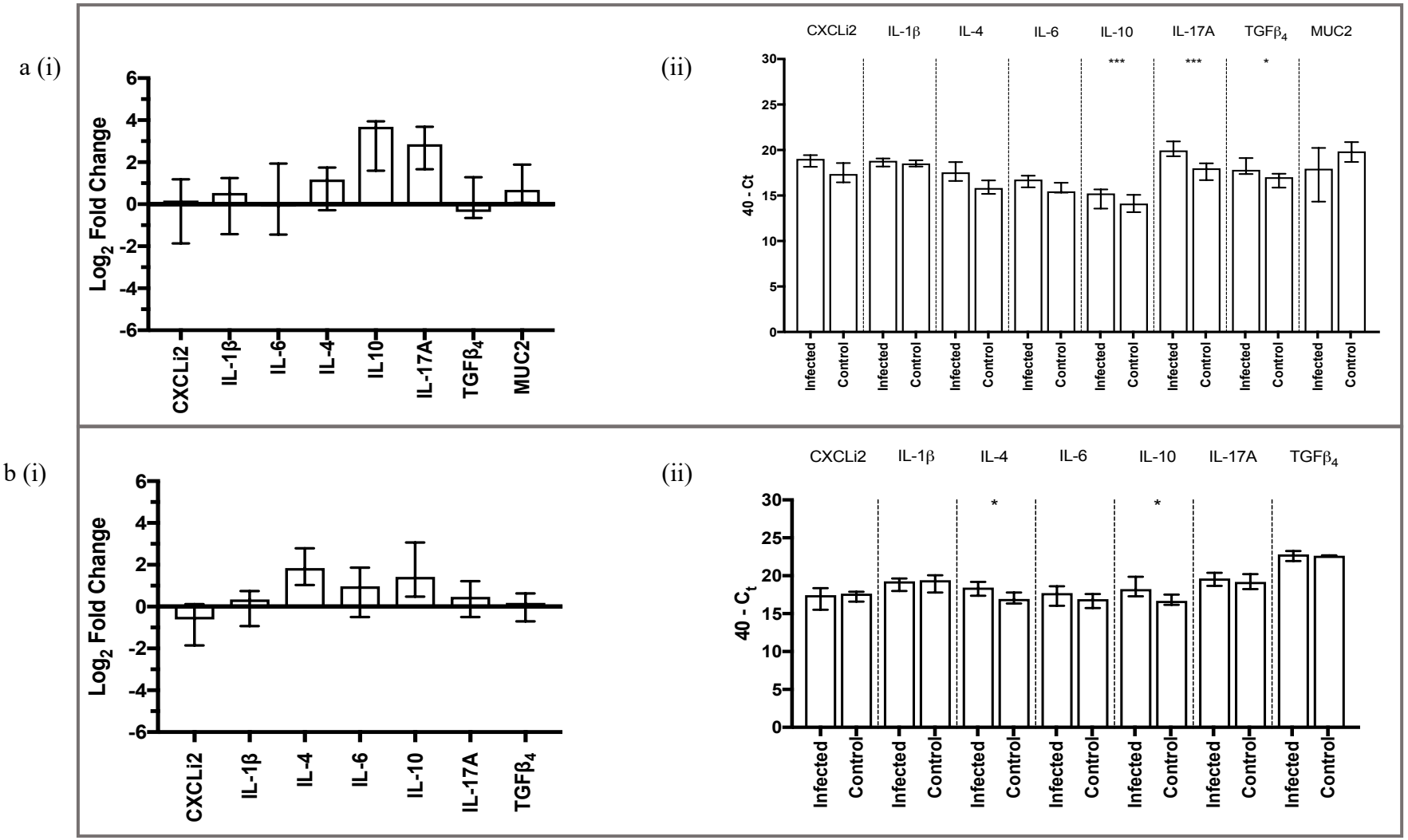


Figure 17. Relative expression (i) and 40 - $^{\Delta}C_t$ (ii) of assessed cytokine, chemokine and AMP transcripts within caecal tissue (a) and caecal tonsil tissue (b) of experimental chickens according to *C. jejuni* challenge status at 28 d.p.i (23 d.p.h). Error bars represent IQR of the median value and statistical significance has been assessed according to 40 - $^{\Delta}C_t$. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as * $p < 0.05$, ***$p < 0.001$. Infected 2 d.p.i n=10, 7 d.p.i n=11, 14, 21 and 28 d.p.i n=12; control 2, 7, 14, 21 & 28 d.p.i n=6.

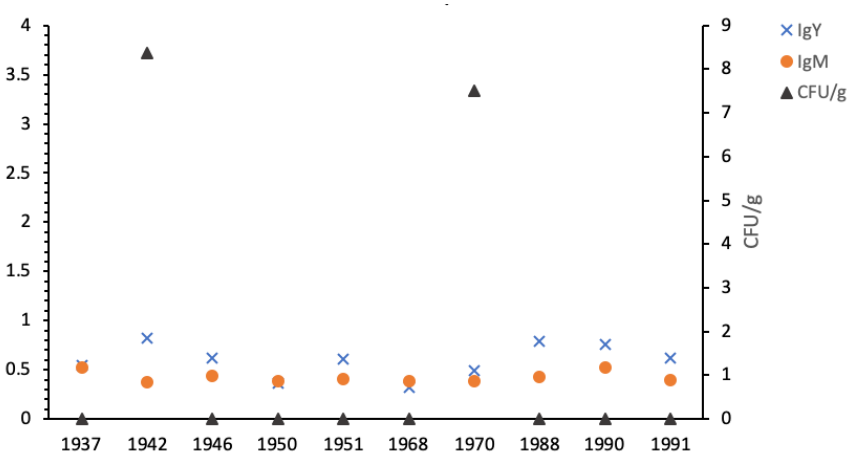
Table 10. Statistical parameters for relative fold change and 40-Ct values for all analysed genes in control and *C. jejuni* infected caecal tissues of chickens at various time-points post infection. All statistical significance values are given for 40-Ct values of gene expression in infected tissues relative to that of control tissues.

	Days post infection														
		2			7			14			21			28		
Challenge status	Gene	40 - Ct; Median (IQR)	P-Value	Fold change; Median (IQR)	40 - Ct; Median (IQR)	P-Value	Fold change; Median (IQR)	40 - Ct; Median (IQR)	P-Value	Fold change; Median (IQR)	40 - Ct; Median (IQR)	P-Value	Fold change; Median (IQR)	40 - Ct; Median (IQR)	P-Value	Fold change; Median (IQR)
Infected	CXCLi2	21.57 (1.41)	0.0002	15.85 (13.23)	21.08 (1.03)	0.002	12.00 (5.63)	19.03 (0.87)	0.0823	2.91 (1.51)	18.56 (0.61)	0.0496	5.25 (2.17)	17.51 (1.89)	1.89	1.08 (1.54)
	IL-1B	20.34 (0.57)	0.0002	6.30(2.56)	20.06 (1.64)	0.001	4.48 (3.05)	18.81 (0.79)	0.6993	2.50 (1.20)	18.55 (1.37)	0.1469	1.89 (1.95)	18.60 (2.19)	2.19	1.45 (1.71)
	IL-4	16.70 (0.41)	0.5135	0.77 (0.25)	18.98 (1.52)	0.0002	5.38 (5.03)	17.32 (1.13)	0.0127	2.66 (1.98)	16.65 (1.83)	0.6993	1.52 (1.81)	16.07 (2.82)	2.82	2.29 (1.96)
	IL-6	20.30 (0.21)	0.0002	5.43 (2.60)	17.56 (1.31)	0.0029	3.40 (2.75)	16.75 (1.11)	0.0637	1.96 (1.27)	17.97 (1.88)	0.0312	2.68 (3.76)	17.61 (1.48)	1.48	0.99 (2.55)
	IL-10	15.11 (2.94)	0.2853	0.88 (1.31)	17.57 (2.34)	0.0503	3.57 (4.78)	15.23 (1.46)	0.2212	1.59 (1.77)	15.98 (1.70)	0.042	8.20 (6.41)	21.01 (2.11)	2.11	12.83 (11.30)
	IL-17A	16.97 (0.84)	0.0513	1.99 (1.25)	17.87 (1.05)	0.0619	3.20 (2.53)	19.95 (1.30)	0.0018	4.79 (5.40)	20.19 (1.50)	0.0559	5.04 (5.91)	20.39 (1.74)	1.74	7.24 (8.58)
	TGFb4	20.84 (0.80)	0.6354	0.95 (0.47)	20.28 (1.51)	0.028	4.57 (3.60)	17.81 (1.42)	0.0193	2.16 (2.82)	18.81 (1.15)	0.0023	1.95 (2.03)	17.16 (1.52)	1.52	0.78 (1.38)
	MUC2	18.85 (2.35)	0.1419	2.26 (2.99)	15.97 (4.31)	0.0007	0.14 (0.15)	17.95 (3.99)	0.1079	0.59 (0.87)	21.13 (1.36)	0.1419	0.64 (0.61)	22.25 (2.62)	0.1812	1.62 (2.04)
Control	CXCLi2	18.04 (2.16)			17.10 (2.17)			17.37 (0.95)			16.58 (1.39)			17.64 (0.71)		
	IL-1B	17.86 (0.49)			17.26 (0.39)			18.52 (0.45)			17.60 (0.22)			18.25 (0.99)		
	IL-4	16.44 (1.03)			16.69 (0.33)			15.82 (0.39)			16.88 (1.23)			16.95 (0.80)		
	IL-6	17.74 (1.52)			16.02 (1.07)			15.46 (0.99)			16.13 (1.42)			16.14 (0.09)		
	IL-10	15.85 (2.85)			15.56 (1.19)			14.12 (0.76)			12.90 (1.64)			15.54 (1.21)		
	IL-17A	15.22 (0.66)			16.82 (0.26)			17.99 (0.80)			17.83 (1.48)			17.49 (0.40)		
	TGFb4	20.92 (0.75)			17.76 (0.58)			17.01 (1.20)			17.00 (1.34)			16.31 (0.44)		
	MUC2	17.48 (3.25)			20.19 (0.90)			19.85 (1.59)			23.33 (1.95)			20.77 (2.41)		

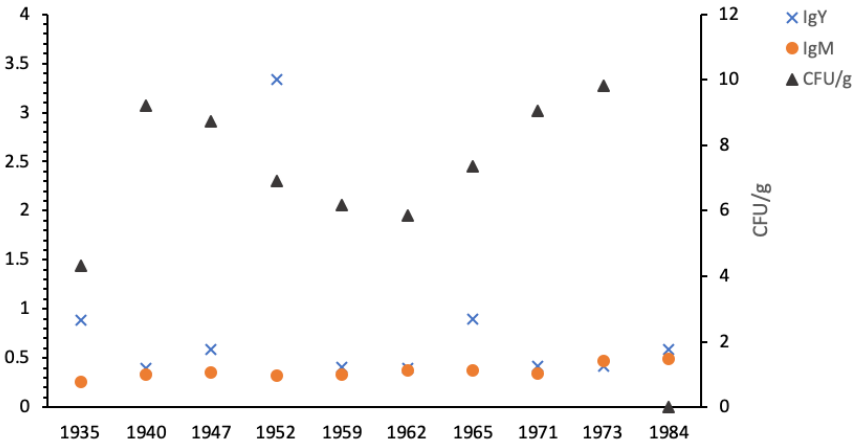
Table 11. Statistical parameters for relative fold change and 40-Ct values for all analysed genes in control and C. jejuni infected caecal tonsil tissues of chickens at various time-points post infection. All statistical significance values are given for 40-Ct values of gene expression in infected tissues relative to that of control tissues.

	Days post infection															
		2			7			14			21			28		
	Gene	40 - Ct; Median (IQR)	P-Value	Fold change; Median (IQR)	40 - Ct; Median (IQR)	P-Value	Fold change; Median (IQR)	40 - Ct; Median (IQR)	P-Value	Fold change; Median (IQR)	40 - Ct; Median (IQR)	P-Value	Fold change; Median (IQR)	40 - Ct; Median (IQR)	P-Value	Fold change; Median (IQR)
Infected	CXCLi2	19.46 (1.06)	0.8887	1.23 (0.87)	18.79 (1.02)	0.1914	2.46 (1.45)	17.18 (1.39)	0.829	0.92 (0.65)	18.56 (0.61)	0.002	2.41 (3.48)	17.44 (2.44)	>0.9999	0.67 (0.75)
	IL-1B	18.44 (1.71)	0.007	0.27 (0.27)	20.00 (0.91)	0.0006	3.46 (2.40)	12.51 (2.16)	0.6835	1.83 (3.22)	17.81 (1.13)	0.2397	1.77 (1.50)	19.25 (1.68)	0.682	1.27 (1.15)
	IL-4	17.77 (2.42)	0.0559	0.41 (0.54)	17.22 (0.58)	>0.9999	1.52 (0.57)	18.27 (0.98)	0.0032	2.24 (2.36)	17.37 (0.84)	0.0182	2.15 (1.56)	18.43 (1.33)	0.5249	3.59 (3.35)
	IL-6	19.02 (2.18)	0.4559	1.20 (2.98)	19.07 (1.40)	0.0005	10.23 (10.86)	16.55 (0.83)	0.9636	0.94 (0.66)	14.83 (1.72)	0.8591	0.75 (1.31)	17.72 (2.36)	0.3011	1.96 (2.64)
	IL-10	19.93 (1.45)	0.3277	1.88 (1.29)	17.37 (0.44)	0.0007	2.46 (0.78)	18.01 (1.75)	0.0031	4.17 (3.44)	17.74 (0.46)	0.028	10.85 (4.75)	18.23 (2.59)	0.0135	2.73 (6.67)
	IL-17A	19.25 (1.06)	0.0017	0.27 (0.19	20.84 (0.75)	0.016	6.59 (5.88)	19.18 (1.34)	0.8916	1.46 (1.01)	17.23 (1.69)	0.3011	2.14 (3.26)	19.63 (1.25)	0.6165	1.39 (1.16)
	TGFb4	22.29 (1.51)	0.0553	2.24 (2.37)	22.77 (0.85)	0.0048	2.84 (1.92)	23.23 (0.70)	0.0144	2.06 (1.34)	21.54 (1.00)	0.6165	0.97 (1.12)	22.81 (1.24)	0.7669	1.14 (0.91)
Control	CXCLi2	19.33 (1.22)			18.09 (2.19)			17.37 (0.95)			16.58 (1.39)			17.64 (0.71)		
	IL-1B	20.15 (1.98)			17.87 (1.42)			11.81 (1.55)			17.14 (0.30)			19.41 (1.50)		
	IL-4	19.64 (1.98)			17.43 (0.87)			17.50 (1.40)			15.06 (0.59)			16.93 (1.08)		
	IL-6	18.88 (0.72)			15.73 (0.49)			16.69 (0.58)			15.75 (1.57)			16.90 (1.21)		
	IL-10	19.52 (1.23)			15.86 (0.88)			15.88 (0.53)			14.62 (3.05)			16.68 (1.04)		
	IL-17A	21.04 (0.63)			18.65 (1.53)			18.77 (1.64)			17.10 (2.75)			19.19 (1.28)		
	TGFb4	24.18 (0.38)			21.42 (0.67)			22.52 (1.15)			21.59 (1.92)			22.65 (0.05)		

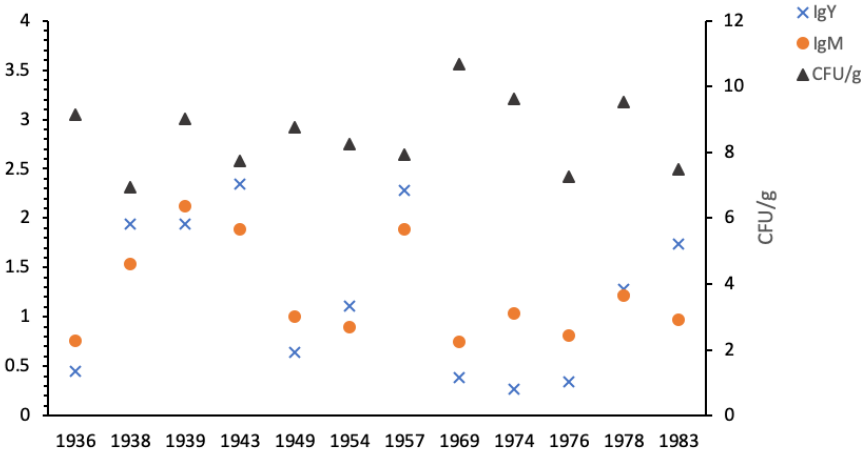
2 d.p.i



7 d.p.i



14 d.p.i



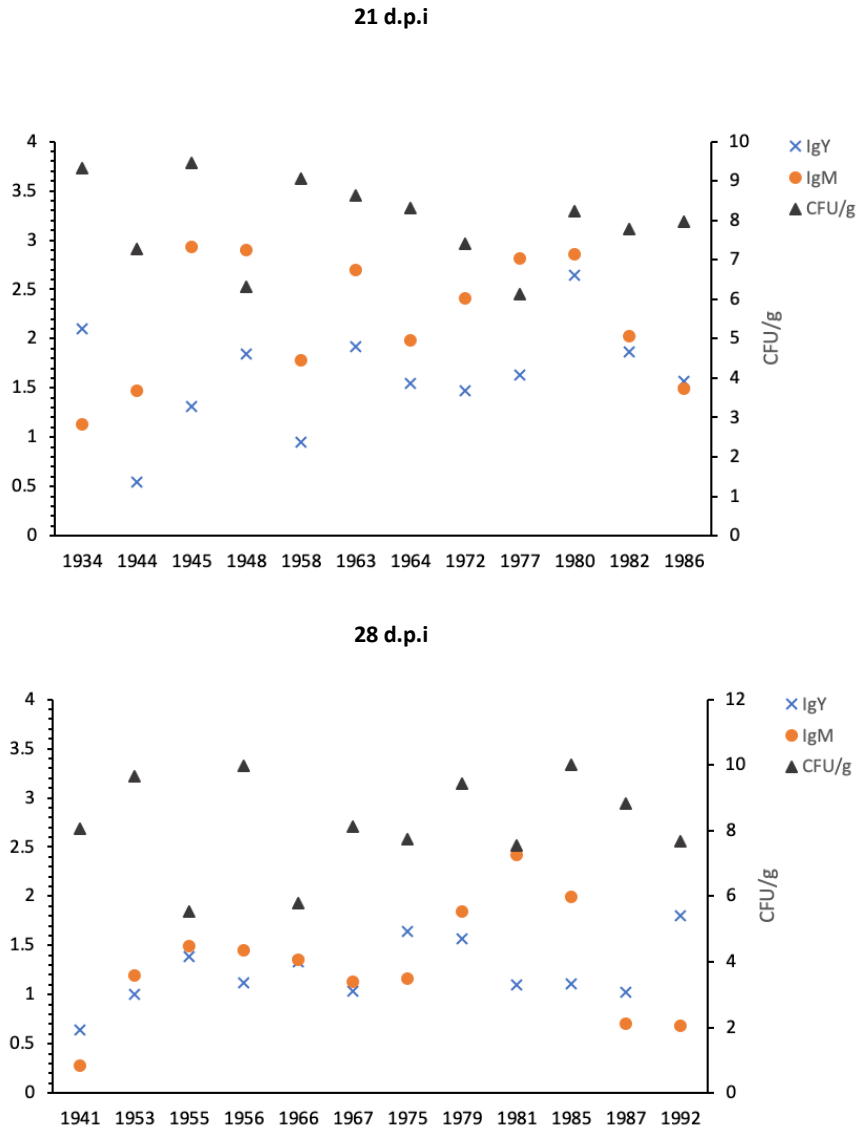


Figure 18. Comparison of IgY and IgM absorbance values following ELISA testing alongside CFU/g of *C. jejuni* within the caecal content of each experimental animal per time point.

Figure 18 highlights potential correlation between caecal *C. jejuni* load and serum IgY and IgM production, as described previously. There appears to be no apparent correlation between the three factors presented.

DISCUSSION

Both experimental infection protocols used within this study highlight the ability of *C. jejuni* to colonise the avian intestinal tract rapidly following ingestion. Although colonisation was apparent in both experimental trials at 2 d.p.i, there was slight disparity between colonisation rate between experiment 1 and experiment 2, being 20 % and 67 % respectively at this time point. Differences between experiments were less apparent by 7 d.p.i, with infected groups from both experimental trials showing between 80 - 90% colonisation. All birds given infection inoculum were positive for *C. jejuni* within the caeca by 14 d.p.i, irrespective of experimental trial. Using similar infective doses of two *C. jejuni* strains (S3B and 21190) to those within our study, Sahin et al. (2003) saw a dramatic increase in percentage of birds shedding *C. jejuni* between 2 d.p.i and 8 d.p.i. By 14 d.p.i, all birds within the work of Sahin et al. (2003) were shedding *C. jejuni* and continued to do so until 28 d.p.i, a characteristic presented within caecal positivity of our study. Caecal colonisation of birds positive for *C. jejuni* across each trial ranged from 5.52 to 10.68 Log_{10} CFU/g in experiment 1 and 5.12 to 8.9 Log_{10} CFU/g in experiment 2. These results are in accordance with the levels of caecal colonisation observed in previous literature, stating a general range of colonisation from 10^6 CFU/g to loads in excess of 10^9 CFU/g (Hermans et al., 2014; Humphrey et al., 2014). This increase in bacterial numbers over time has also been evident in other studies (Hansson et al., 2010) and linked to the increase in flock risk of colonisation with flock age (Bouwknegt et al., 2004). Increases in *C. jejuni* colonisation such as those described here will almost certainly increase the risk of human campylobacteriosis from the broiler chicken reservoir as high proportions of *C. jejuni* positive caecal samples or high bacterial numbers within these contents increase the numbers of *Campylobacter* on chicken carcasses (Hansson et al., 2010). It is important to consider that while routinely described as highly motile, no confirmation of motility was performed during this research. With flagellar-mediated motility central to *C. jejuni* colonisation, future work should incorporate such methodologies as standard practice prior to infection. Work by de Vries et al (2015) highlights the importance of such methodologies and defines how this can be conducted through inoculation of solid growth medium with the bacterium and subsequently recording the diameter of the ring of bacterial growth following incubation.

Although obvious trends in dynamics of colonisation existed across both trials, variation in both positivity status and subsequent *C. jejuni* load existed within experimental animals at specific time points of each experimental trial. This variation was particularly evident at earlier time points prior to 14 d.p.i. Since all birds of each trial were inoculated with the same

infectious dose of *C. jejuni* at the same time point, differences in infection dynamic between individuals reflects how homogenous infection dynamics are rarely associated with *C. jejuni* infection and that individuals within the same flock can exhibit largely dissimilar susceptibility to infection (Hansson et al., 2010).

In conjunction with caecal colonisation, *C. jejuni* colonisation was often detected within the ileal tissues of birds from both experimental trials. While it is generally accepted that the predominant site for *C. jejuni* colonisation of the chicken is within the caecal crypts, as with this work, reports of its colonisation throughout the intestinal tract are prevalent (Hermans et al., 2014; Humphrey et al., 2014; Lin, 2009). When directly comparing caecal *C. jejuni* burden with that of the ileum, this temporal variation in colonisation was of similar magnitude between experiments 1 & 2, with caecal colonisation being 2.74 and 2.59 fold higher respectively. Work by Beery et al. (1988) supports this finding that *C. jejuni* primarily colonizes distal regions of the GIT, principally the caecum. The blind ended caeca represent a region of the GIT with a relatively low flow rate and as such, high retention time of ingested material (Savage, 1977). In contrast, the ileum is a straight section of intestinal canal with a much higher rate of flow and more dynamic environment (Johansen et al, 2006). This stable environment within the caecal crypts denotes a site easily colonised by *C. jejuni*, and subsequently a site that makes colonisation difficult to clear. Further to this, Johansen et al. (2006) has demonstrated how infection with *C. jejuni* potentially alters the microbial communities within the caeca, a function not found following infection within the ileum. Consequently, it may be that the anatomical structure of the intestinal site provides primary influence on the ability of *C. jejuni* to establish colonisation, while persistence within these niches once established might be the results of a combination of both anatomical structure and alterations in the host microbiome resulting from this first pathogenic colonisation.

Extending from the established colonisation of sites throughout the GIT, this study further confirms the ability of *C. jejuni* to colonise systemic sites, far beyond that of the GIT. Colonisation of splenic tissue occurred as early as 2 d.p.i within experiment 1 and shortly after at 7 d.p.i in experiment 2. Within both experimental trials, splenic infection always preceded hepatic infection. There appeared to be no apparent correlation between the frequency of extra-intestinal *C. jejuni* identification and *C. jejuni* burden within the GIT. While of relatively recent discovery, this dissemination of *C. jejuni* to systemic tissues has previously been identified in the work of Vaezirad et al. (2017), whereby *Campylobacter* was identified in the

blood and liver tissues of broiler breeds, with this being subsequently linked to intrinsic invasive abilities of specific *Campylobacter* strains, alongside disparities in the susceptibility of chicken lines to such invasion. While further corroborated in the work of others (Chaloner et al., 2014; Humphrey et al., 2014), the means by which *Campylobacte*r is able to navigate the intestinal barrier is still undefined. Knudsen et al. (2006) suggests that the very colonisation characteristics of *Campylobacter* species, whereby they interact heavily with the intestinal microbiota allow for a close interface with the intestinal epithelial surface and, as such, an increased prospect for invasion and spread to distant tissues. Arguments of this nature provide further weight to the notion that *C. jejuni* infection within the broiler chicken may not be solely commensal as once suggested, but instead able to induce immune responses outside that of a tolerogenic nature. Our study shows maximal frequency of colonisation of extra-intestinal tissues occurred from 21 d.p.i onward, the common slaughter age of most commercial broiler chickens. While current *C. jejuni* control strategies within poultry systems are generally focused on reducing caecal *C. jejuni* load, this work suggests concomitant importance of other tissues, such as the liver and spleen, in providing a source of carcass contamination post-slaughter (Reich et al., 2008). *C. jejuni* contamination of poultry liver tissues offers not only a means of contamination for outer carcass tissue, but also acts as a direct source for human campylobacteriosis. As identified by Whyte et al. (2006), poultry liver tissues are commonly consumed by people worldwide. Internal and external contamination of liver tissues with *C. jejuni* is often identified, which, considering the common cooking method of leaving such liver tissues 'pink in the middle', makes chicken liver tissue an important risk factor in itself for human exposure (Whyte et al., 2006). Despite showing high *C. jejuni* colonisation at post-mortem, no obvious clinical signs of disease were present in either experimental study. This lack of clinical disease has been reported widely in current literature, however Humphrey et al. (2014) has stated evidence of diarrhoea within fast growing poultry breeds following bacterial infection. The ubiquitous nature of *C. jejuni* within the poultry industry alongside its lack of recognizable signs of infection make bacterial control within poultry farming ever more arduous.

This study further confirmed the induction of inflammatory responses within the caecal tissues of a commercial broiler chicken line following exposure to *C. jejuni* infection, with this showing strong time-dependent succession. While median expression values have been used to represent specific transcript expression per-time point according to challenge status, magnitude of response varied according to individual animal within each treatment group.

Reflecting the lack of conformity in *C. jejuni* colonisation patterns *in vivo*, this variable immune response supports such understanding that both pathogen infection dynamics alongside host immunological responses are not unilateral. Changes in the chemokine ligand CXCLi2 alongside IL-1β and IL-6 associated with *C. jejuni* infection were found in the caecal tissue as early as 2 d.p.i. IL-1β and IL-6 are known pro-inflammatory cytokines with IL-1β acting as part of an innate immune response able to potently induce the upregulation of both IL-6 and CXCLi2 (Reid et al., 2016). Together IL-1β and IL-6 are vital in early stimulation of Th17 responses, a pathway previously highlighted as being a predominant cytokine response to *C. jejuni* infection within the broiler chicken (Reid et al., 2016). This may be supplementing this with an early influx of heterophils likely induced from pro-inflammatory CXCLi2 upregulation, being a highly homologous ligand to human IL-8 (Kogut et al., 2005). While caecal tonsil tissues also showed tendency toward an early pro-inflammatory response with an early surge in IL-1β expression, this was instead accompanied by IL-17A upregulation. Reid et al. (2016) has previously linked increases in IL-1β expression with subsequent IL-17A upregulation as a mechanism of protection against experimental *C. jejuni* challenge. At 7 d.p.i, all analysed cytokine and chemokine gene transcripts following *C. jejuni* infection showed some magnitude of upregulation in both caecal and caecal tonsil tissues compared to tissues of non-infected animals. While the pro-inflammatory response, initiated early post-infection continued to dominate, IL-4 upregulation became apparent within the caeca. It could be postulated that an early increase in IL-6 expression induced an increase IL-4 response whereby a Th2 mediated response could be initiated (Avery et al., 2004). Interestingly, although many cytokine transcripts appear upregulated within the caecal tonsil 7 d.p.i, it is those associated with anti-inflammatory regulatory responses (IL-10 & TGFβ_4) that appear most dissimilar to samples from non-infected birds, with such a response continuing through to 28 d.p.i. This sequential decline in pro-inflammatory inducers has been previously associated with subsequent increase in anti-inflammatory response within *Salmonella* infection as a means of preventing exaggerated inflammatory responses within the intestinal tissue to infecting bacterium (Salisbury, 2012). It is also of interest that expression of the innate immune glycoprotein Mucin2 showed strong downregulation within the caecal tissue of infected birds at this time-point. While true *in vivo* response of secreted and surface mucins to *C. jejuni* challenge remains uncharacterized, use of RT-qPCR techniques in work by (Tu et al., 2008) has shown how Mucin2 may in fact act as an environmental prompt for *C. jejuni* to modulate expression of genes associated with colonisation and pathogenicity.

Production of specific IgY, IgM and total secretory IgA in response to *C. jejuni* infection varied according to the level of prolonged persistence. For both serum IgY and IgM, peak titres were reached at 21 d.p.i, although this was not correlated with any significant reduction in *C. jejuni* colonisation within the caeca. The rise in *Campylobacter* specific IgY and IgM has previously been understood to begin approximately 2-3 weeks post experimental infection, and so our results are not thought to be dissimilar to previous conjecture (de Zoete et al., 2007). IgM are associated with low-affinity binding with high avidity to an array of self and non-self antigens. With a range of activities, including the regulation of immune response and induction of further antibody mediated activity against bacterial and viral infection, the induction observed here is likely indicative of primary immune response to the experimental *C. jejuni* infection. While IgY is able to utilize complement and Fc mediated macrophage uptake as a means of direct bacterial clearance, sIgA instead acts primarily through receptor blockade and immune exclusion mechanisms (Mantis et al., 2011). Generally, little is known regarding exact immune exclusion mechanisms attributed to sIgA, but consensus largely refers to agglutination, mucus entrapment and mechanistic clearance preventing pathogenic interaction with the intestinal epithelium (Mantis et al., 2011). Our data shows significant increases in sIgA of *C. jejuni* challenged birds above that of non-challenged from 21 d.p.i, with this trend accompanied by a general upregulation of Treg association cytokine subsets, being IL-10 and TGFβ_4 within caecal tonsil tissues. IL-10 and TGFβ_4 have been suggested as important inducers of class switching processes modifying B cell production from IgM to IgA production within chickens (de Vries et al., 2014). Although *Campylobacter*-specific sIgA has previously been association with protection against disease in humans (Janssen et al., 2008), the increases in total sIgA shown within this study indicate that this may only become apparent toward commercial slaughter age, making their influence on general poultry *C. jejuni* burden negligible, as indicated in work by Lacharme-Lora et al. (2017) . Such findings, notably those regarding sIgA, would benefit from further corroboration principally due to the variation observed within the titres recorded for birds acting as controls. This variation between individuals of the same group may limit the biological relevance of the findings described since these largely rely on comparison to control individuals.

The results from this study have provided a foundation of understanding in the colonisation dynamics and host immune response to *Campylobacter* that can be built upon throughout the remaining work demonstrated within this thesis. We have exhibited how the infection biology of *C. jejuni* within our broiler trial data sets represent much of that within currently available

literature (Awad et al., 2018; Lin, 2009; Newell, 2002). This involves rapid colonisation of the intestinal tract, with high levels of bacterial load within the caecal contents at a level which remains sustained until at least commercial slaughter age (Hermans et al., 2014). Bacterial load was lower within other sites of the intestinal tract outside of the caecal crypts. Isolation of *C. jejuni* from two systemic sites provides evidence of the bacteria being able to diffuse outside of the GIT, and into systemic tissues (Awad et al., 2018; Chaloner et al., 2014; Humphrey et al., 2014). This dissemination into sites distant from the intestine appears to increase alongside general *C. jejuni* infection persistence. We have generated data sets providing supporting theory that *C. jejuni* infection within the chicken is not simply commensal as previously described and is capable of generating an array of inflammatory immune pathways largely centralized around Th17 responses. Colonisation of multiple tissues such as the liver will more than likely make it increasingly difficult to prevent carcass *C. jejuni* contamination and also limit bacterial entry into the human food chain. There were no indications of obvious disease within any animals included within either study, in direct disparity to that seen in human campylobacteriosis (Newell, 2002). Oral infection methodologies used here provide a relatively accurate infection model due to the fecal-oral route of infection found in almost all field flock infections.

Chapter Three: *Caecal Microbiota Transplant in broiler chickens*

INTRODUCTION

Campylobacter infection remains the most common cause of global bacterial foodborne gastroenteritis (de Zoete et al., 2007). The acute gastroenteritis resulting from the majority of human *Campylobacter* infections is self-limiting, although can infrequently manifest into sequelae indications including arthritis and Guillain-Barré syndrome (GBS) (Acheson & Allos, 2001; Pasquali et al, 2011). As previously highlighted, both campylobacteriosis and any subsequent indication presents an economic burden to both the worlds most developed and deprived countries. A consequence of low infectious dose requirements for human infection, prevalence of *Campylobacter* in more than 80 % of UK retail poultry carcasses and carcass contamination often in excess of several thousand bacteria per cm^2 skin, has made poultry meat a reservoir of infection with huge public health implication (Corry & Atabay, 2001).

To implement effective infection control strategies, identifying the high-risk phases of the overall 'farm-to-table' production system within the poultry industry is essential (Hayama et al., 2011). Some focus has been placed on the impact of carcass processing at slaughter houses on the entry of *Campylobacter* into the human food chain (Hayama et al., 2011). Although much emphasis is placed on carcass contamination from bird feathers and introduction of intestinal content (Berrang et al., 2004), results from Chapter 2 highlight the ability of a multitude of tissues, both within the alimentary canal and more distant, to harbour *Campylobacter*. Cross contamination of *C. jejuni* bacteria to previously non-positive broiler carcasses during poultry processing post-slaughter has been reported at over 75% within slaughterhouses across Europe (Meunier et al., 2016) and will have influence on whole production flocks processed on a single line (Hayama et al., 2011). Hayama et al. (2011) show how it is not only initial flock prevalence of *Campylobacter* that can be a problem at this stage of production, but also the total *Campylobacter* load of each contaminated carcass. Interventions, in place to limit the frequency of carcass contamination during processing post slaughter, are often constrained by this factor. To illustrate this understanding, when mean carcass *Campylobacter* load was identified as 2.7 Log_{10} CFU/carcass, the frequency of *Campylobacter* carcass contamination across that flock post processing was lower than that prior to processing (Hayama et al., 2011). However, with a higher initial carcass contamination of 6.7 Log_{10} CFU/carcass, flock *Campylobacter* cross-contamination was considerably higher, resulting in a significant increase in prevalence post-processing (Hayama et al., 2011). As such, both *Campylobacter* prevalence and total colonisation load are of significant importance in controlling contamination of poultry meat destined for the human food chain. To mitigate the

risk of human exposure, implementing control strategies to reduce both load and prevalence of *Campylobacter* in the primary, on-farm production stage are seen as the most effective measures (Meunier et al., 2016).

Multiple on-farm control strategies have been considered as a means of controlling *C. jejuni* levels in poultry production (Ghunaim, 2009). Refinements in biosecurity processes has been heralded as one of the most effective methods of *C. jejuni* control that can be implemented at farm level and throughout this phase of production. In theory, a reduction in the initial exposure of poultry to *Campylobacter* through comprehensive biosecurity practices should be an effective means of intervention (Lin, 2009). However, intervention strategies employing varying levels of biosecurity practices have reported mixed success (Pasquali et al., 2011). It is suggested by Wagenaar et al. (2006), that for improvements in biosecurity to truly have any further impact on *Campylobacter* levels within poultry flocks, the current understanding of both infection sources, and the risk factors mitigating these must first be refined. While considerable effort has been afforded to deriving an effective vaccination strategy to reduce *Campylobacter* infection of the chicken, results have been largely contradictory, with no reputable model developed (de Zoete et al. 2006; Meunier et al. 2017; Kobierecka et al. 2016; Hermans et al. 2014). With understanding of the avian immune response to *Campylobacter* still in its infancy, developing an effective vaccination strategy to the point of commercial realisation requires the navigation of multiple obstacles (Pasquali et al., 2011). One particularly problematic challenge is the short life span of commercial broiler chickens (~ 6 weeks of age) and the initial presence of maternally derived antibodies (until ~ 2-3 weeks of age) (Lin, 2009). Together, these factors result in a relatively short phase in the broiler lifecycle within which a strong antibody response to *Campylobacter* infection must be mounted (Lin, 2009). It should also be considered that, depending on the vaccination strategies utilized, this short timespan must also encompass any withdrawal period necessary.

Therapeutic administration of bacteriophages or bacteriocins to chickens already showing *C. jejuni* colonisation has been demonstrated as a potential replacement for widespread antimicrobial use (Lin, 2009). Working in accordance with the intestinal mucosal barrier, bacteriophages and bacteriocins show a range of antibacterial properties and prove particularly promising modulators of the intestinal environment in both humans and animals (Lopetuso et al., 2019). However, links to *Campylobacter* resistance to both therapies have been suggested (Carrillo et al., 2005; Stern, 2008).

Since the chicken gut microbiome acts as an immediate biological barrier against *C. jejuni*, it's manipulation through pre- and probiotics has been thought to play an essential role in *Campylobacter* reduction and control in commercial poultry production. Therapeutic manipulation of the microbiota in production of livestock has an extensive history (Borody, et al., 2013). Animal husbandry practices including transfaunation, the transfer of rumen content in cattle are well established therapies for common indications. Incorporation of the use of dietary products, particularly probiotics alongside other microbial interventions, to manipulate or modify poultry intestinal microbiomes are becoming increasingly popular within the commercial poultry industry (Gupta et al., 2016). The food and agriculture organisation of the united nations (FAO) adopted definition of a probiotic formulation is "live microorganisms which when administered in adequate amounts confer a health benefit to the host", with these often being administered via a digestive route (FAO/WHO, 2001). Early work using avian intestinal flora to reduce *Salmonella* colonisation in chicks by (Rantala & Nurmi, 1973) was the forerunner of many subsequent studies on probiotics. However, the basis of how any manipulation of the microbiota is effective in reducing pathogen load within the chicken remains unclear. Forming the basis of current literature, common theory would indicate that there are two broad hypotheses; firstly, any microflora preparation may have a competitive exclusion (CE) effect, originally an ecological term, based around competition for a niche and resources. We also now understand that intestinal tract bacteria such as *Firmicutes* produce metabolites, such as butyrates, that can inhibit the growth of *Proteobacteria* (Eeckhaut et al., 2011). Secondly, probiotics and microflora preparations may drive immune development and immunity in the gut, helping limit pathogen colonisation and as such, decrease host disease susceptibility (Stanley et al., 2014). While somewhat promising data exists on the efficacy of probiotics against specific gastrointestinal tract (GIT) illness in veterinary species, attempts at reproducing and refining this for use against *C. jejuni* colonisation of the modern broiler chicken has been largely empirical in nature, with little evidence of a practical industrial role (Oakley et al. 2014; Stanley et al. 2014). Recommended doses of oral probiotics will often deliver a relatively low magnitude of viable microorganisms compared to that found within the native microbiota (Borody & Khoruts, 2012). In addition, although often initially derived from the avian intestinal tract, many probiotic bacterial strains have undergone extensive environmental adaptation during *ex vivo* culture, potentially limiting its ability to establish within the complex intestinal niche of the broiler chicken (Borody & Khoruts, 2012). Considering these aspects alone, probiotic cultures often exhibit

only transient influence on the intestinal microbiota and will generally require repeat administration (Borody & Khoruts, 2012).

Often, a probiotic preparation is of relatively trivial bacterial taxonomic complexity when compared to that of the developed chicken caecal microbiome. As such, it may be conceptually more pertinent to consider the gut as an entire system, with multiple interwoven facets as oppose to the distinct sum of individual bacterial entities. The introduction of a complete, stable gut microbiota from a healthy donor into a recipient through a Faecal Microbiota Transplant (FMT) has recently been incorporated into the therapeutic treatment of an array of known and idiopathic conditions (Borody & Khoruts, 2012). Perhaps the best described and most effective clinical use of FMT in human medicine is to treat recalcitrant *Clostridium difficile* infection (CDI), a result of dysbiosis stemming from antibiotic use, is one of the most notable examples of current therapeutic applications. A study by Aas et al. (2003) presented a FMT treatment success rate exceeding 90% within trial evaluable patients, such findings being reproducible throughout considerable further research (Agrawal et al., 2016; Kelly et al., 2016). Although the scientific rationale behind its efficacy remains somewhat elusive, the undoubtable success of FMT in the treatment of CDI warrants further indication of multiple applications beyond current practice. While use of FMT is becoming progressively disseminated throughout human clinical practice, FMT in a modern sense has not yet been adopted into livestock.

In contrast to many animal species, the large intestine of the poultry GIT comprises a pair of blind ended caecal sacks, a unique feature strongly developed within the domesticated chicken. Comparable to other regions of the poultry GIT, the caecal microbiome has long been correlated with poultry health and productivity. Feeding on from the ileum, the ceca is the main site of fermentation for undigested foodstuffs, being emptied only once every 12 - 24 hours (Clavijo & Flórez, 2018). Potentially interlinked with this extended food retention time, the caeca are often described as having the most taxonomically diverse and abundant microbiota across all chicken GIT sites (Clavijo & Flórez, 2018). Additionally, with the caecum being the primary site of *Campylobacter* species colonisation, transplantation of caecal microbiota in replacement of the more commonly utilised faecal microbiota may offer unique benefits in chicken gut health, productivity and susceptibility to disease.

Here we transfer a caecal microbiota from healthy, *Campylobacter*-free control donor chickens directly to newly hatched chicks. The aims of all in vivo challenge models used here were to assess the effect of CMT on host susceptibility to subsequent *C. jejuni* infection and its transmission within an experimental flock. From this we were looking to devise practice guidelines of donor selection, Caecal Microbiota Transplant (CMT) material selection and preparation and route of administration.

MATERIALS AND METHODS

BACTERIAL ISOLATES AND GROWTH CONDITIONS

Strain *Campylobacter jejuni* M1 was used as the infecting inoculum, prepared as previously described in Chapter 2. Serial 10 - fold dilutions of the final Mueller Hinton Broth (MHB [Lab M Ltd., Heywood, Lancashire, UK]) liquid culture were made in 1 x Maximum Recovery Diluent (MRD [Lab M Ltd, Bury, UK]) to 10^{-8} for viable colony enumeration via Miles & Misra (1938) and plated onto Colombia Blood Agar (CAB [Lab M Ltd., Heywood, Lancashire, UK]) supplemented with 5 % defibrinated horse blood (Oxoid, Basingstoke, Hampshire, UK) as described in Chapter 2 before incubation for 48 hours at 41.5°C.

CAECAL MICROBIOTA TRANSPLANT (CMT) PREPARATION

Since CMT has not been widely tested, a lack of standard practice guidelines exists for the preparation of caecal material for transplantation (Borody & Khoruts, 2012). All trials listed within this work were conducted using CMT material prepared as described here. Samples of 10 g caecal content were aseptically collected at point-of-cull from five uninfected control animals used within experiment 1 (Chapter 2) and pooled to create a sample of 50 g. Caecal content was immediately snap frozen at collection using liquid nitrogen to prevent the deterioration of transplantation material and prolonged aerobic exposure. This pooled CMT material was stored at - 80°C until further processing.

Stored CMT material was subsequently thawed in a warm water bath set at 37°C before being diluted 1:20 (w/v) in sterile 1 x Phosphate Buffered Saline (PBS [Lab M Ltd, Heywood, Lancashire, UK]) and filtered through a coarse 25 µM Whatman® (Sigma, Poole, Dorset, UK) filter to achieve desired CMT consistency. Diluted CMT was vortexed for 1 minute to ensure thorough content dispersal before aliquoting and storage at - 80°C until use. At point of use,

2 mL aliquots of CMT (1:20) were warmed in a water bath at 37°C until fully thawed. Thawed CMT material was vortexted for 1 minute before being delivered to recipient chicks within 1 - hour of thawing. Treatment was delivered to all chicks using a 1 mL sterile syringe (Fisher Scientific, Loughborough, UK) through a sterile oral gavage (Sigma, Poole, Dorset, UK).

MALDI-TOF MS BIOTYPER SAMPLE PREPARATION

A Sterilin sterile cotton swab (ThermoFisher Scientific, Loughborough, UK) containing prepared CMT inoculum was spread onto CAB agar ensuring to cover the entire agar surface, replicating this process a total of nine times. Of these replicates, three were incubated in either aerobic, microaerobic or anaerobic conditions for a period of 48 hours.

Following incubation, plates were observed for morphologically distinct colony growth, with each differentially identified colony being sub-cultured on CAB agar and incubated for a further 48 hours in their respective growth conditions. Each identified colony was smeared as a thin film directly onto a spot on a stainless steel MALDI target plate (Bruker Daltonic, Billerica, MA, USA). Samples were then overlain with 1 µl of 70 % formic acid and allowed to air dry at room temperature. Immediately, 1 µl of α-cyano-4-hydroxycinnamic acid matrix (HCCA), prepared according to manufacturers' instruction, was overlain and allowed to air dry at room temperature. All further processing was conducted by the Veterinary Diagnostic Microbiology Service, University of Liverpool, using standard operating procedures for Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) biotyper analysis.

EXPERIMENTAL ANIMALS

All work was conducted in accordance with United Kingdom legislation governing experimental animals under project license PPL 40/3652 and was approved by the University of Liverpool ethical review process prior to the award of this license. All animals held at this site were checked a minimum of twice daily to ensure individual animal health and welfare. Description of experimental housing condition, feed and unit biosecurity measures can be found in Chapter 2, as previously described by (Humphrey et al., 2014).

For all experimental trial groups requiring chicks to be hatched within our experimental unit, embryonated Ross 308 hens' eggs were obtained from a commercial hatchery and transported directly to the University of Liverpool high biosecurity experimental unit. All eggs were inspected for shell quality and those with catastrophic damage were discarded from the experiment. All eggs were dipped for 2 - 3 minutes in a 1 % (1:100 dilution) solution of Ambicide™ (PatrickPinker, Latteridge, Bristol, UK) maintained at 38 – 41 °C. Eggs were subsequently air dried and wiped with 1 % peracetic acid (Sigma, Poole, Dorset, UK) before transfer to a sterile incubator. Eggs were incubated for 21 days at 37.7 °C in an automatic roll incubator (Brinsea, Milton Keynes, UK) and candled 7 days after setting to ascertain viability. Only viable eggs were retained for the remainder of the trial. Relative humidity was maintained at 45 - 55 % until day 18 of incubation where the humidity increased to 60 - 70 % until hatching.

OVERVIEW OF INDIVIDUAL EXPERIMENTAL TRIAL DESIGNS

The key aims underpinning each trial within this study are described below in addition to being detailed in Table 12. For full detail on all experimental animal housing, protocols for inoculum preparation and administration, and post-mortem processing, see Chapter 2.

Table 12. Key experimental protocol time points for experimental challenge models 3 – 6

	Experiment 3	**Experiment 4**	**Experiment 5**	**Experiment 6**
CMT Inoculation	Immediate	Immediate	7 d.p.h	Immediate
***C. jejuni* challenge model**	Seeder	Seeder	Seeder	Direct
***C. jejuni* challenge dose**	10^6 CFUml^{-1}	10^6 CFUml^{-1}	10^6 CFUml^{-1}	10^4 CFUml^{-1}
Experimental groups	CMT (n=22) Ext. Control (n=24)	CMT (n=8) Ext. Control (n=12)	CMT (n=12) Ext. Control (n=17)	CMT (n=20) Ext. Control (n=24)
Swabbing time points (d.p.i)	2, 5, 8, 12	3, 5, 7, 10	3, 5, 7, 10	7, 10
Cull (d.p.i)	14	12	12	4, 10

Experimental design – Experiment 3

A total of 22 chicks were successfully hatched following 21 days of incubation, with these receiving 0.1 – 0.2 ml prepared CMT inoculum within 4-hour post-hatch as previously described. At point of hatch, a further 24 age-matched day-old Ross 308 chicks were obtained from the same commercial hatchery and received 0.1 – 0.2 ml sterile 1 x PBS within 4 hours

of arrival. Treatment groups were housed in separate rooms with lobbied entry and additional dedicated protective clothing and boots. At 14 days post-hatch (d.p.h) all animals were confirmed to have *Campylobacter* negative status. At 21 d.p.h, two randomly selected birds from each group were orally infected with 0.2 mL 10^6 CFU/mL *C. jejuni* in MHB via oral gavage.

Cloacal swabs were collected from all birds at multiple time-points post infection to assess within-group *C. jejuni* shedding. Swabs were collected from all birds at 23 (2 d.p.i), 26 (5 d.p.i), 29 (8 d.p.i) and 33 (12 d.p.i) days post-hatch. At 35 d.p.i (14 d.p.i) all birds were culled via cervical dislocation. Aseptic collection of splenic & liver tissues and caecal & ileal content was conducted as described in Chapter 2. Figure 19 provides a visual explanation of key experimental time-points.

Experimental design – Experiment 4

This experiment was designed to act as a repeat experimental study of Experiment 3 with the aim of validating the initial results obtained.

A total of 8 chicks were successfully hatched following 21 days of incubation, with these receiving 0.1 – 0.2 ml prepared CMT inoculum within 4 hours post hatch as previously described. At point of hatch, a further 12 age-matched day-old Ross 308 chicks were obtained from the same commercial hatchery and received 0.1 – 0.2 ml sterile 1 x PBS within 4 hours of arrival. Treatment groups were housed in separate rooms with lobbied entry and additional dedicated protective clothing and boots. At 14 d.p.h, all animals were confirmed to have *Campylobacter* negative status. At 21 d.p.h, two randomly selected birds from each group were orally infected with 0.2 ml 10^6 CFU/ml *C. jejuni* in MHB via oral gavage.

Cloacal swabs were collected from all birds at multiple time-points post infection to assess within-group *C. jejuni* shedding. Swabs were collected from all birds at 24 (3 d.p.i), 26 (5 d.p.i), 28 (7 d.p.i) and 31 (10 d.p.i) days post hatch. At 33 d.p.i (12 d.p.i) all birds were culled via cervical dislocation. Blood samples were collected via cardiac puncture immediately post-cull before aseptic collection of splenic & liver tissues and caecal & ileal content was conducted as described in Chapter 2. Figure 20 provides a visual explanation of key experimental time-points.

Experiment 3 - Effect of CMT on seeder C. jejuni infection;

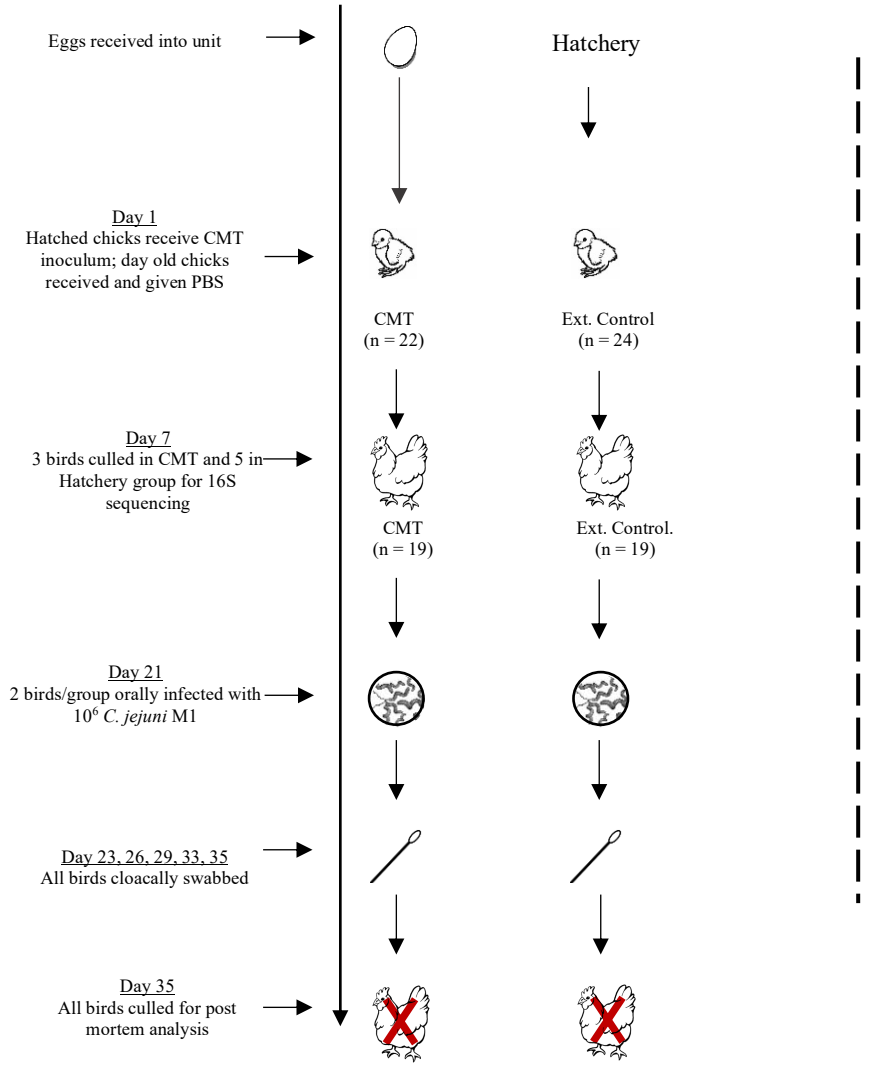


Figure 19. Key time-points associated with experimental trial 3

Experiment 4 – Effect of CMT on seeder C. jejuni infection (Repeat);

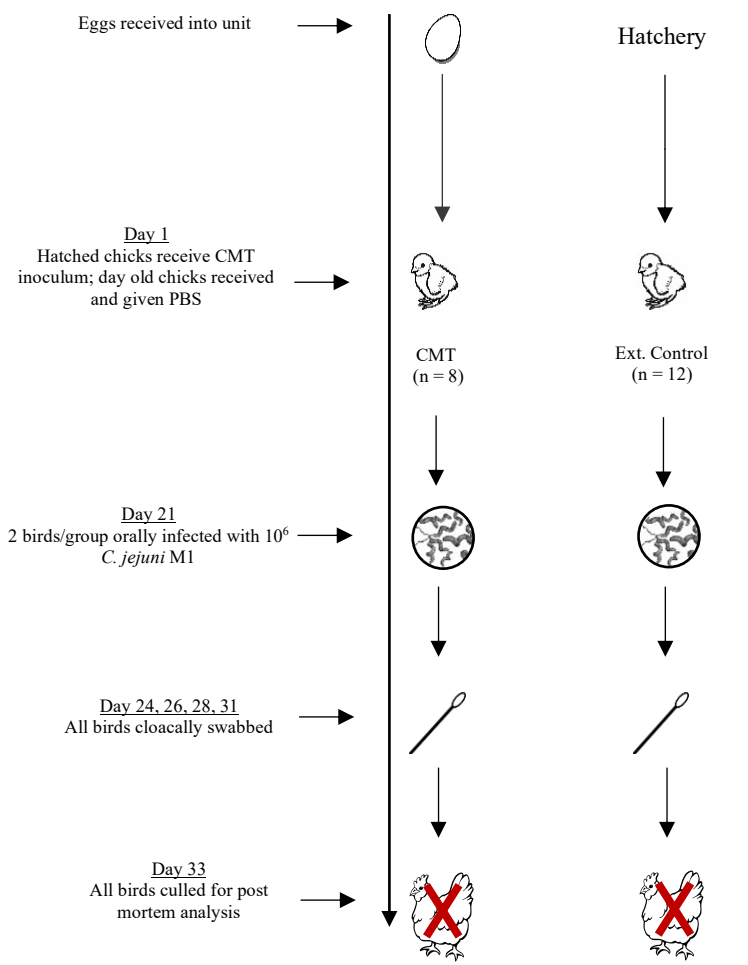


Figure 20. Key time-points associated with experimental trial 4

Experimental design – Experiment 5

The aim of this trial was to understand how time of CMT administration will impact its efficacy by delaying CMT delivery to 7 d.p.h. Age matched, 1 day-old mixed sex chicks (n=32) of Ross 308 broiler chickens were obtained from the same commercial hatchery as in used in all previous experimental trials and transported directly to the University of Liverpool high-biosecurity experimental poultry unit. At 7 d.p.h, birds were divided into two treatment groups, with one being inoculated with 0.1 – 0.2 ml prepared CMT (n=15) and the other sterile 1 x PBS (n=17). From this point on, treatment groups were housed within separate rooms with lobbied entry and additional dedicated protective clothing and boots. At 14 d.p.h, all animals were confirmed to have *Campylobacter* negative status. At 21 d.p.h, two randomly selected birds from each group were orally infected with 0.2 mL 10^6 CFU/ml *C. jejuni* in MHB via oral gavage. Cloacal swabs were collected from all birds at multiple time points post infection to assess within-group *C. jejuni* shedding. Swabs were collected from all birds at 24 (3 d.p.i), 26 (5 d.p.i) and 28 (7 d.p.i) and 31 (10 d.p.i) days post hatch. At 33 d.p.i (12 d.p.i) all birds were culled via cervical dislocation. Aseptic collection of splenic & liver tissues and caecal & ileal content was conducted as described in Chapter 2. Figure 21 provides a visual explanation of key experimental time-points.

Experimental design – Experiment 6

The aim of this trial was to understand CMT efficacy against direct doses of C. jejuni, where level of infection is not determined by within-flock transmission. 20 chicks were hatched following 21 days of incubation, with these receiving 0.1 – 0.2 ml prepared CMT inoculum within 4 hours post-hatch as previously described. At point-of-hatch, a further 24 age-matched day-old Ross 308 chicks were obtained from the same commercial hatchery and received 0.1 – 0.2 ml sterile 1 x PBS within 4-hours of arrival. At 14 d.p.h, all animals were confirmed to have *Campylobacter* negative status. At 21 d.p.h, all birds from each group were orally infected with 0.2 mL 10^4 CFU/ml *C. jejuni* in MHB via oral gavage. On day 25 (4 d.p.i), 22 birds were culled via cervical dislocation for post-mortem analysis (CMT n = 10; Ext. Control n = 12). Cloacal swabs were collected from all remaining birds at 28 (7 d.p.i) and 31 (10 d.p.i) days post hatch to assess within-group *C. jejuni* shedding. At 31 d.p.h (10 d.p.i) all remaining birds were culled via cervical dislocation. For all post-mortem analyses, samples of splenic and liver tissues & caecal and ileal content were collected aseptically as described in Chapter 2. Figure 20 provides a visual explanation of key experimental time-points.

Experiment 3 - Effect of delayed CMT;

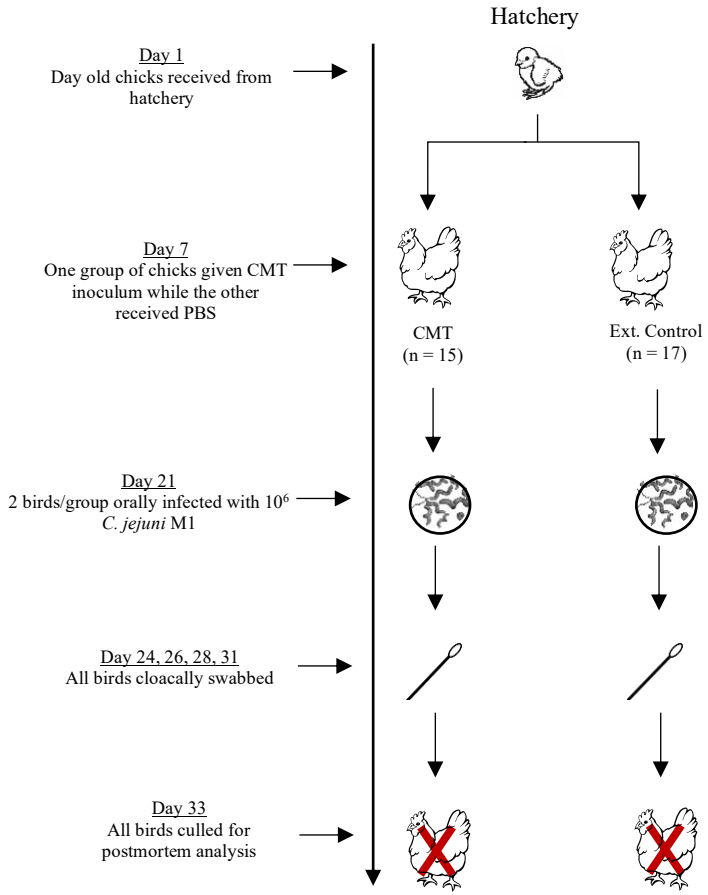


Figure 19. Key time-points associated with experimental trial 5

Experiment 4 – Effect of CMT on direct C. jejuni infection;

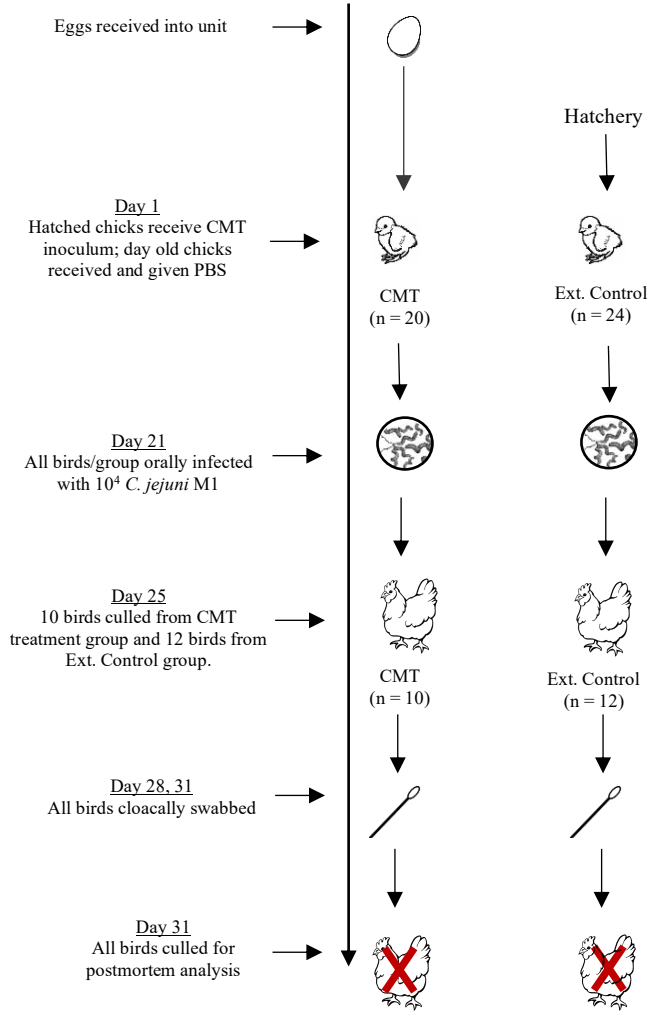


Figure 20. Key time-points associated with experimental trial 6

CLOACAL SWABBING

Cloacal swabs were collected in all described trials as a means of assessing within-group *C. jejuni* shedding. Table 1 provides further detail on swabbing time-points according to each experimental protocol. Swabs were streaked onto selective blood-free agar (mCCDA [modified charcoal-cefoperazone-deoxycholate agar supplemented with *Campylobacter* enrichment supplement SV59]) (Mast Group Ltd, Bootle, Merseyside, UK) in a way to cover the entire agar surface before incubating at 41.5°C for 48 hours in microaerobic conditions. The cotton tip of each swab was subsequently used to inoculate 2 mL of Exeter enrichment broth (1100 mL nutrient broth, 55 mL lysed defibrinated horse blood, *Campylobacter* enrichment supplement SV59 [containing trimethoprim (10mg/L) and amphotericin B (2mg/L); Mast Group Ltd, Bootle, UK] and *Campylobacter* growth supplement SV61 [containing sodium pyruvate (250mg/L), sodium metabisulphate (250mg/L) and ferrous sulphate (250mg/L); Mast Group Ltd]) and incubated at 41.5°C for 48 hours in microaerobic conditions. Following enrichment, samples were vortexed and plated onto mCCDA using a 3 µl loop. All plates were incubated at 41.5°C for 48 hours in microaerobic conditions before being assessed for *C. jejuni* positivity. Cloacal swabs were processed within 2 hours of sample collection.

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism version 7.00 for Mac OS X (GraphPad Software Inc., San Diego, USA). Prior to further statistical analysis, all bacterial enumeration data was first assessed for normality using a D'Agostino-Pearson omnibus normality test. Data sets showing non-normal distribution ($p < 0.05$) were further assessed for significance using non-parametric Mann Whitney-U testing to compare ranks, with significance set at $p < 0.05$. For those data sets showing normal Gaussian distribution, parametric unpaired t-tests were used with significance set at $p < 0.05$.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Post-mortem blood samples were aseptically collected via cardiac puncture from all birds within experiment 3 at 35 days post-hatch (14 d.p.i). All blood samples were centrifuged at 13000 x *g* for 5 minutes before serum collection and storage at - 20 °C for subsequent ELISA

analysis. All samples were assessed for *Campylobacter* specific serum immunoglobulin-G (IgY) and IgM levels through ELISA protocols described in Chapter 2.

RESULTS

MALDI-TOF MS BIOTYPER IDENTIFICATION

Culture of CMT inoculum onto CAB agar yielded a total of 34 morphologically distinct colonies when assessed visually. Using MALDI-TOF MS biotyping, taxonomic assignments were able to classify 28 of these into 15 taxonomic species. Species identification was assigned a unique score classification generated by comparing the peak profile (or peptide mass fingerprint) of each tested strain against known database entries, quantifying similarity on a logarithmic score between 0 and 3 (Richter et al., 2012). Only species identifications with a score value of ≥ 1.7 were included in analysis as being highly probable (Richter et al., 2012). Full MALDI Biotyper identification results and associated logarithmic score values are provided in Appendix 2 with only unique taxonomies by growth condition shown in Table 13.

Table 13. Culture conditions and bacterial taxonomic identification following MALDI TOF biotyper analysis. All presented species were identified with a representative score value of ≥ 1.7

Culture media	Culture conditions	Number of unique species identified	Identified organism
CAB	Aerobic	10	*Bacillus altitudinis*
			Bacillus cereus
			Bacillus pumilus
			Bacillus subtilis
			Enterococcus faecium
			Enterococcus faecalis
			Escherichia coli
			Lactobacillus paracesei
			Solibacillus silvestris
			Staphylococcus cohnii
	Microaerobic	10	*Bacillus amyloliquefaciens*
			Bacillus amyloliquefaciens_ssp_plantarum
			Bacillus cereus
			Bacillus megaterium
			Bacillus mojavensis
			Bacillus pumilus
			Bacillus subtilis
			Clostridium perfringens
			Enterococcus faecium
			Escherichia coli
	Anaerobic	2	*Clostridium perfringens*
			Enterococcus faecium

EXPERIMENTAL TRIALS 3 & 4

Cloacal shedding

Between 2 d.p.i and 12 d.p.i, cloacal swabs were used to determine the dynamics of *C. jejuni* infection within each population of birds. Looking first at experimental seeder models used in experiment 3 and 4, transmission of *C. jejuni* was considerably delayed within the CMT group of both experiments compared to that of respective External hatchery treatment groups (Figure 21).

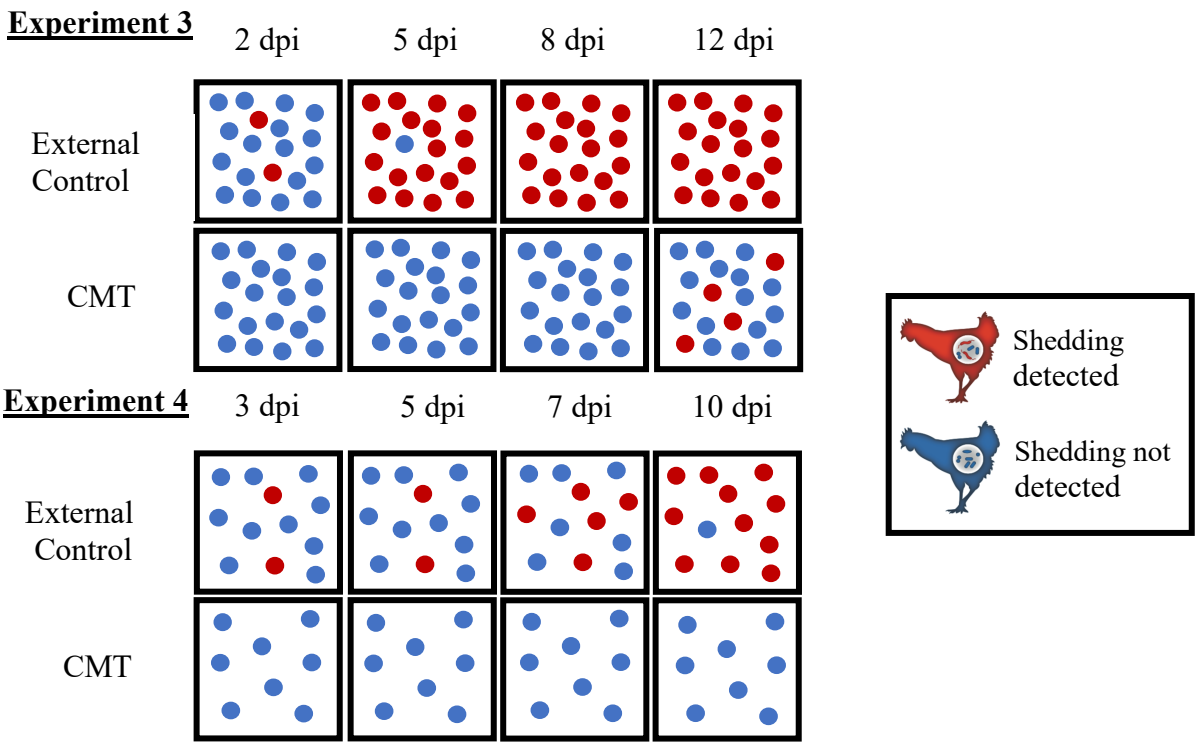


Figure 21. *C. jejuni* M1 transmission within treatment groups of experimental broiler chickens. Cloacal shedding was determined through cloacal swabbing at pre-defined time points according to experimental protocol. Red shapes depict birds detected as shedding *C. jejuni* while blue shapes show groups with no detected bacterial shedding. Experiment 3: CMT (n=19), External control (n=19); Experiment 4: CMT (n=8), External control (n=12).

By 2 d.p.i, 2/19 birds (94 %) within the External control group of experiment 3 were shedding *C. jejuni*, with 19/19 birds (100 %) being *C. jejuni* positive by 8 d.p.i. In contrast, no cloacal shedding of *C. jejuni* was detectable within the CMT group of experiment 3 until 12 d.p.i, whereby only 4/19 birds (21 %) were *C. jejuni* positive. The transmission dynamics within experiment 3 were similar to that of experiment 4, however CMT treated birds of experiment 4 showed no detectable *C. jejuni* shedding during the course of the trial. Shedding was detected within the hatchery group from 3 d.p.i (2/12 [17 %]) and by 10 d.p.i, 11/12 birds (92

%) were *C. jejuni* positive. Results are provided in Figure 22 and Figure 23 detailing *C. jejuni* detection pre- and post- enrichment of cloacal swabs for all experimental birds of experiment 3 and experiment 4 respectively.

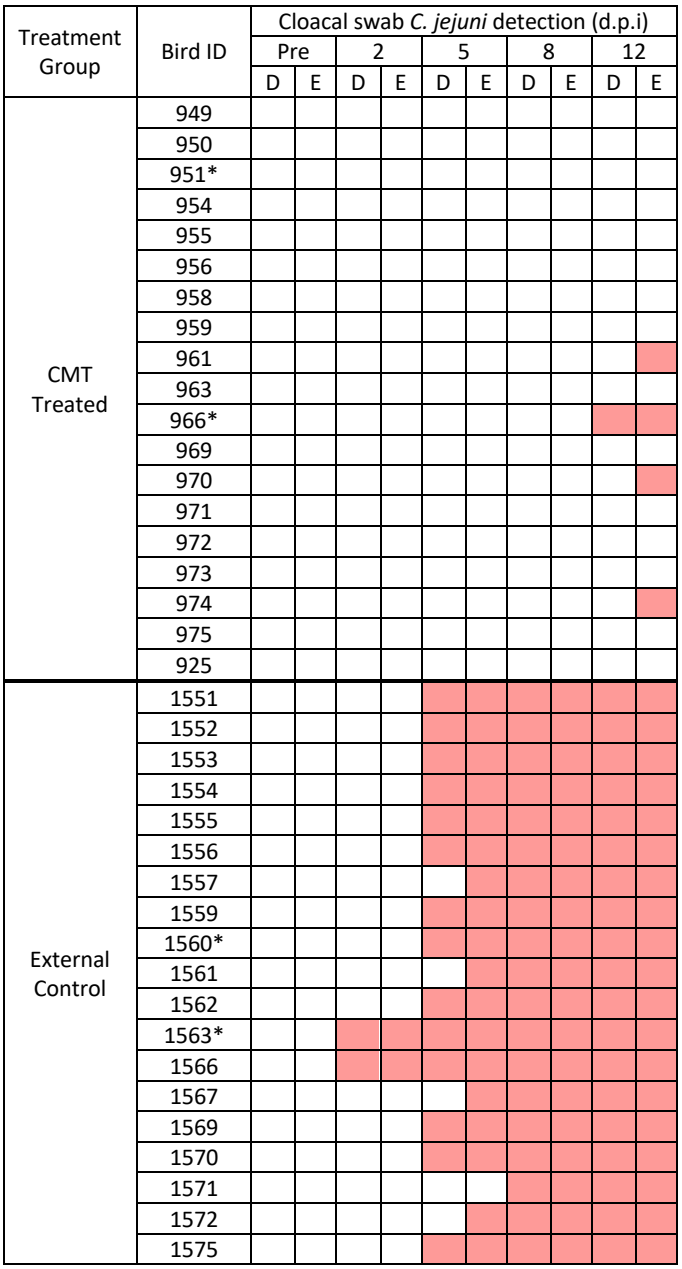


Figure 22. Detection of *C. jejuni* via cloacal swabbing at time points stipulated in experiment 3 protocols. Red squares depict *C. jejuni* detection within a single swab sample, whereby 'D' indicates results are from direct plating of swab and 'E' depicts results are from enriched samples. All birds showing '*' were directly infected as seeder birds as part of experimental protocols listed. CMT (n=19); Ext. control (n=19).

Treatment Group	Bird ID	Cloacal swab *C. jejuni* detection (d.p.i)									
		Pre		3		5		7		10	
		D	E	D	E	D	E	D	E	D	E
CMT Treated	1801										
	1802										
	1803										
	1804*										
	1805*										
	1806										
	1808										
	1809										
External Control	3751*										
	3752										
	3753										
	3754										
	3755*										
	3756										
	3757										
	3758										
	3759										
	3760										
	3761										
	3762										

Figure 23. Detection of *C. jejuni* via cloacal swabbing at time points stipulated in experiment 4 protocols. Red squares depict *C. jejuni* detection within a single swab sample, whereby 'D' indicates results are from direct plating of swab and 'E' depicts results are from enriched samples. All birds showing '*' were directly infected as seeder birds as part of experimental protocols listed. CMT (n=8); Ext. control (n=12).

Caecal colonisation

Caecal content was aseptically collected from all birds in experiment 3 and experiment 4 at 35 d.p.h (14 d.p.i) and 33 d.p.h (12 d.p.i) respectively. Early caecal microbiota transplantation significantly reduced *C. jejuni* M1 load within caecal content compared to non-treated External control birds using seeder bird infection models (Figure 24). Enumeration data collected detailing *C. jejuni* load within caecal content of birds from both experiment 3 and experiment 4 showed non-normal distribution ($p < 0.05$) and as such, will be discussed and presented as medians including their respective IQR.

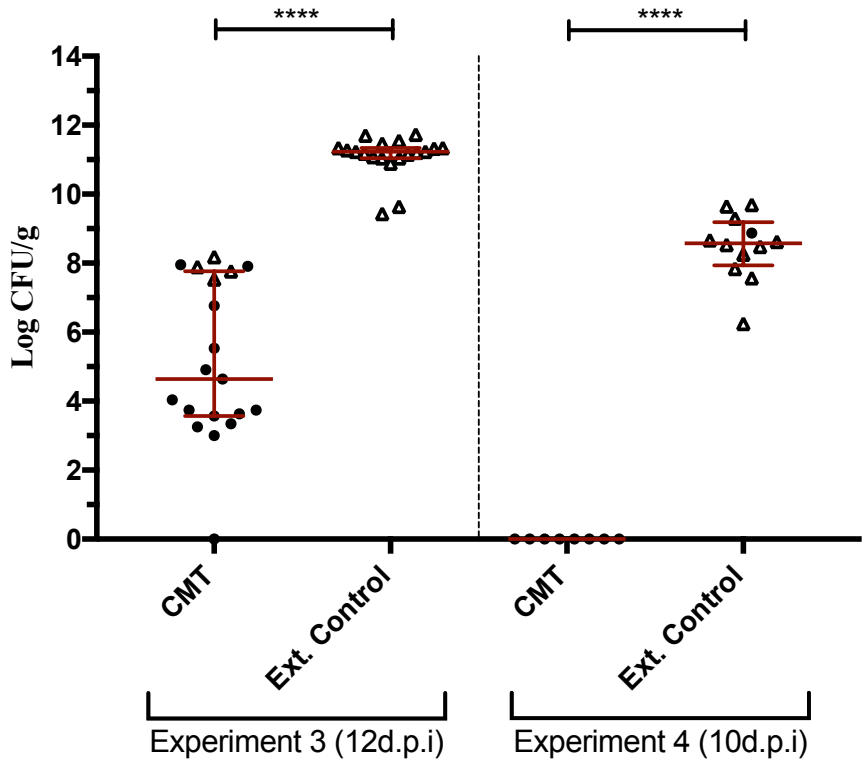


Figure 24. Levels of *C. jejuni* M1 in the caeca of broiler chickens under experimental conditions based on protocols listed for experiment 3 (CMT n=19; Ext. control n=19) and experiment 4 (CMT n=8; Ext. control n=12). Each symbol represents caecal *C. jejuni* load for an individual animal. Statistical analysis per treatment group are given as median values with associated IQR with significance determined using Mann Whitney-U analysis. Levels of significance are given as ****p<0.0001. All birds detected as shedding are given reported using a hollow triangle symbol, while all birds not detected as shedding *C. jejuni* are reported using a circle.

All birds within the External control groups of both experiment 3 and experiment 4 showed caecal *C. jejuni* colonisation at point-of-cull, with median Log_{10}CFU/g values of 11.23 (IQR 0.3) and 8.58 (IQR 1.25) respectively. *C. jejuni* was detected in the caecal content of all but one (18/19; 95 %) CMT treated birds within Experiment 3 with median detected load of 4.78 (IQR 4.18) Log_{10}CFU/gram, although this was significantly lower than that detected for External control birds ($p < 0.0001$). Similarly, experiment 4 showed significant protective ability of CMT treatment against *C. jejuni* colonisation compared to External control birds ($p < 0.0001$), with no detectable colonisation of *C. jejuni* within birds treated with CMT material.

Ileal colonisation

Samples of ileal content were collected at post-mortem from each bird of both experiment 3 and experiment 4 at 35 d.p.h (14 d.p.i) and 33 d.p.h (12 d.p.i) respectively. As with caecal colonisation, early CMT reduced ileal colonisation within both experimental trials 3 & 4

compared to non-treated External control birds using seeder bird infection models (Figure 25). Enumeration values collected for *C. jejuni* load within ileal content of birds from both experiment 3 and experiment 4 showed non-normal conformation ($p < 0.05$) and as such, these results will be discussed and presented as medians, including their respective interquartile range.

Within both trials described, ileal colonisation occurred at both a lower frequency and, when found, to a lower burden than that found within the caeca. In both experiment 3 and experiment 4, no ileal *C. jejuni* colonisation was detected in any bird treated with CMT. The External control group of experiment 3 showed 15/19 (79 %) birds to be colonized with *C. jejuni* within the ileum, with group median colonisation of 3.6 Log_{10}CFU/gram (IQR 1.47). Ileal colonisation of the External control group of experiment 4 showed 5/12 birds (42 %) were *C. jejuni* positive, although median colonisation remained at 0.00 Log_{10} CFU/g (IQR 0.58) for this treatment group. Appendix 2 details caecal and ileal enumeration data.

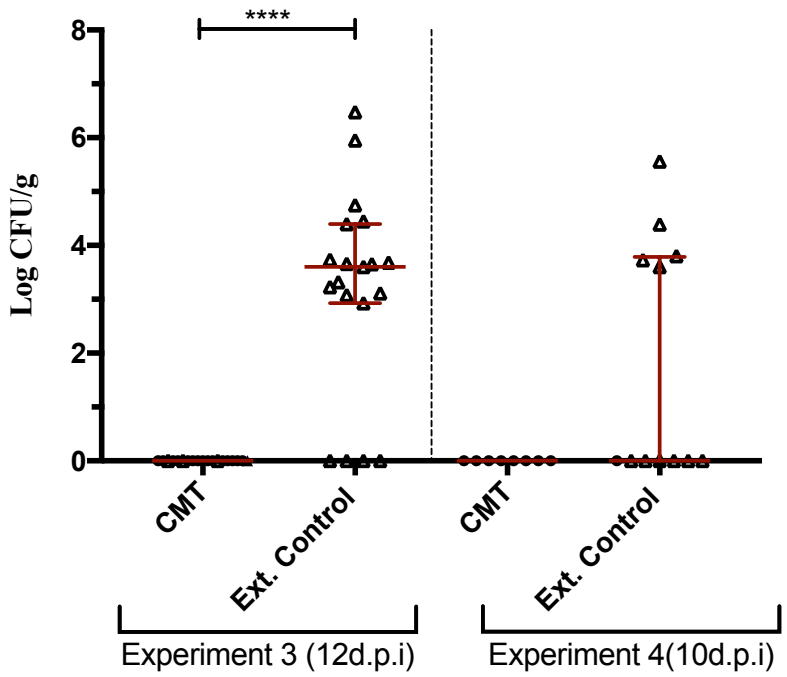


Figure 25. Levels of *C. jejuni* M1 in the ileum of broiler chickens under experimental conditions based on protocols listed for experiment 3 (CMT n=19; Ext. control n=19) and experiment 4 CMT n=8; Ext. control n=12). Each symbol represents ileal *C. jejuni* load for an individual animal. Statistical analysis per treatment group are given as median values with associated IQR with significance determined using Mann Whitney-U analysis. Levels of significance are given as ****p<0.0001. All birds detected as shedding are given reported using a hollow triangle symbol, while all birds not detected as shedding *C. jejuni* are reported using a circle.

Extra-intestinal spread of *C. jejuni*

At post-mortem examination, extra intestinal *C. jejuni* colonisation was present in both experimental replicates 3 and 4. Experiment 3 showed *C. jejuni* within the liver tissue of 2/19 (11 %) External control birds and 1/19 (5 %) CMT birds. This result was similar in experiment 4, with *C. jejuni* present in 2/12 (17 %) liver and 1/12 (8 %) spleen samples from External control birds while no *C. jejuni* colonisation was seen within the CMT population of this experimental model. Figure 26 shows spleen and liver *C. jejuni* detection per bird for experiment 3 and 4 respectively according to their unique identification number pre and post sample enrichment.

(a)

Treatment Group	Bird ID	*C. jejuni* positivity			
		Spleen		Liver	
		D	E	D	E
CMT Treated	949				
	950				
	951*				
	954				
	955				
	956				
	958				
	959				
	961				
	963			■	■
	966*				
	969				
	970				
	971				
	972				
	973				
	974				
	975				
	925				
External control	1551				■
	1552				
	1553				
	1554				
	1555				
	1556				
	1557				
	1559				
	1560*				
	1561				
	1562				
	1563*				
	1566				
	1567				
	1569				
	1570				
	1571				
	1572			■	■
	1575				

(b)

Treatment Group	Bird ID	*C. jejuni* positivity			
		Spleen		Liver	
		D	E	D	E
CMT Treated	1801				
	1802				
	1803				
	1804*				
	1805*				
	1806				
	1808				
	1809				
External Control	3751*				
	3752			■	■
	3753				
	3754				
	3755*				
	3756				
	3757		■		
	3758				
	3759			■	■
	3760				
	3761				
	3762				

Figure 26. Detection of *C. jejuni M1* within liver and splenic tissues of broiler chickens under experimental conditions based on protocols listed for (a) experiment 3 (CMT n=19; Ext. control n=19) and (b) experiment 4 (CMT n=8; Ext. control n=12). Red squares depict *C. jejuni* detection within a single sample, whereby 'D' indicates results are from direct plating of tissue homogenate and 'E' depicts results are from enriched samples.

EXPERIMENTAL TRIAL 5

Cloacal shedding

To assess the impact of 7 d.p.h CMT administration on efficacy against *C. jejuni* transmission, swabs taken between 24 d.p.h (3 d.p.i) and 31 d.p.h (10 d.p.i) during experimental model 5. Here, we saw a slight delay in transmission of *C. jejuni* within the CMT population compared to that of the External control group, however protection against colonisation was not sustained (Figure 27).

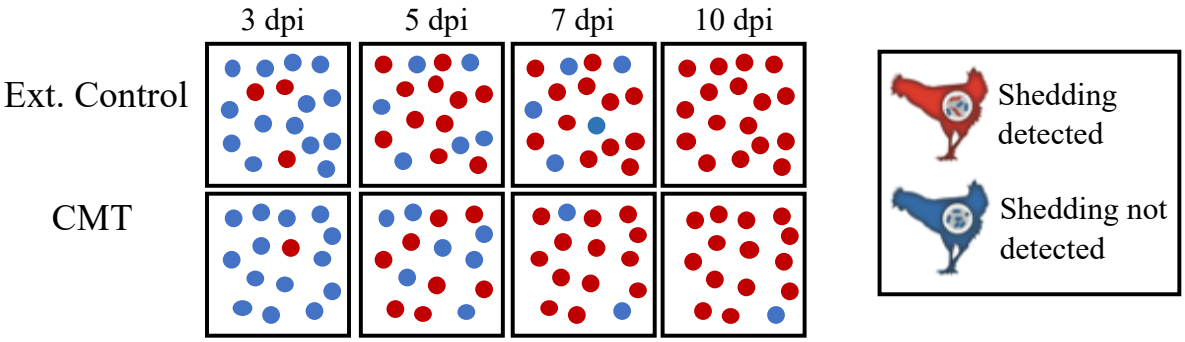


Figure 27. *C. jejuni* M1 transmission within treatment groups of experimental broiler chickens. Cloacal shedding was determined through cloacal swabbing at pre-defined time-points according to experimental protocols. Red shapes depict birds detected as shedding *C. jejuni* while blue shapes show groups with no detected bacterial shedding. CMT n=15; Ext. control n=17.

At 3 d.p.i 1/15 birds (7 %) was shedding *C. jejuni* within the group of birds given the CMT while this number was 3/17 (18 %) within the External control group. By 5 d.p.i, the difference in frequency of shedding between the two groups was negligible, with this relationship continuing until swabbing at 10 d.p.i whereby all birds in both groups were shedding *C. jejuni* in almost all individuals; 14/15 birds (93 %) from the CMT treatment group and 17/17 (100 %) from the External control group. Swab results for experiment 5 are provided in Figure 28, showing *C. jejuni* detection pre- and post-enrichment according to unique individual bird ID number.

Treatment Group	Bird ID	Cloacal swab *C. jejuni* detection (d.p.i)									
		Pre		3		5		7		10	
		D	E	D	E	D	E	D	E	D	E
CMT Treated	2251										
	2252										
	2253										
	2254										
	2255*										
	2256										
	2257										
	2258										
	2259										
	2260										
	2261										
	2262*										
	2263										
	2264										
	2265										
External Control	1776										
	1777										
	1778										
	1779										
	1780										
	1781										
	1782										
	1783										
	1784										
	1785										
	1786										
	1787										
	1788*										
	1789										
	1790										
	1791										
	1792*										

Figure 28. Detection of *C. jejuni* via cloacal swabbing at time points stipulated in experiment 5 protocols (CMT n=15; Ext. control n=17). Red squares depict *C. jejuni* detection within a single swab sample, whereby 'D' indicates results are from direct plating of swab and 'E' depicts results are from enriched samples. All birds showing '*' were directly infected as seeder birds as part of experimental protocols listed.

Caecal colonisation

Caecal content was collected 33 d.p.h (12 d.p.i) from all birds of experiment 5, with all birds in both treatment groups showing caecal positivity for *C. jejuni* (Figure 29). Enumeration values collected for caecal *C. jejuni* load showed normal conformation following Log_{10} transformation ($p > 0.05$) and as such, these results will be discussed and presented as mean values, including their respective standard deviation values. Sample processing errors occurred during post-mortem affecting one experimental animal of the External control treatment group for this experiment (Bird ID: 1787) and there are no data corresponding to

post-mortem samples for this experimental animal. Treatment group sizes were modified to n= 15; CMT administration 7 d.p.h and n=16; External control for all tissue sample data sets pertaining to this experiment

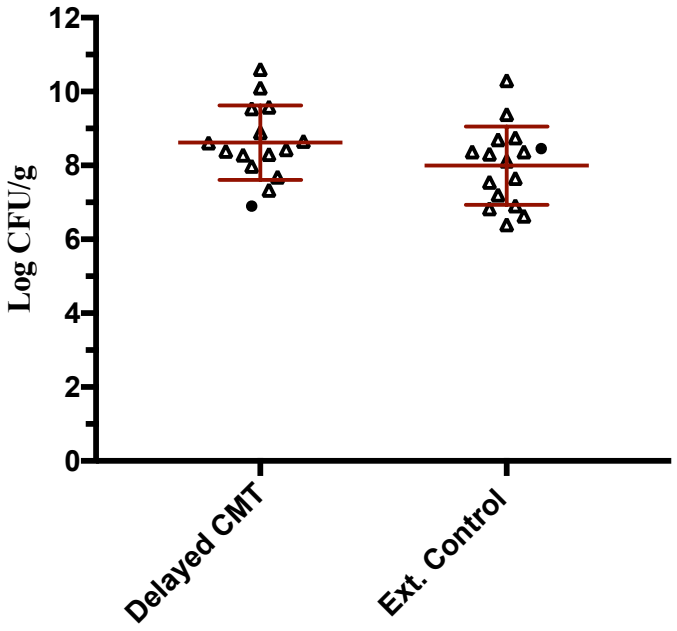


Figure 29. Levels of *C. jejuni* M1 in the caeca of broiler chickens under experimental conditions based on protocols listed for experiment 5 (CMT n=15; Ext. control n=16). Each symbol represents caecal *C. jejuni* load for an individual animal. Statistical analysis treatment group are given as median values with associated IQR with significance determined using Mann Whitney-U analysis. All birds detected as shedding are given reported using a hollow triangle symbol, while all birds not detected as shedding *C. jejuni* are reported using a circle.

All birds in both the External control treatment group and the treatment group receiving 7-day CMT administration had detectable *C. jejuni* colonisation of the caeca, with mean Log_{10}CFU/gram values of 8.00 (± 1.06) and 8.60 (±1.01) respectively. CMT administration delayed until 7 days of age, as applied within this protocol, resulted in no significant difference in final caecal *C. jejuni* colonisation between treatment groups (p = 0.1041).

Ileal colonisation

Ileal colonisation with *C. jejuni* was both less frequent and less pronounced as that seen with caecal colonisation and previous experiments. Unlike trends described with early delivery of CMT inoculum, 7 d.p.h CMT administration had no effect on frequency of ileal *C. jejuni*

colonisation or final ileal *C. jejuni* burden (Figure 30). Enumeration values collected for *C. jejuni* load within ileal content of birds from Experiment 5 showed non-normal conformation ($p < 0.05$) and as such, these results with be discussed and presented as medians, including their respective interquartile range.

Of the birds given CMT 7 d.p.h, 6/15 birds (40 %) had *C. jejuni* present within the ileum at point of cull, with this being 6/16 (38 %) of the External control group. No significant variation was seen between treatment groups in ileal burden of *C. jejuni* ($p = 0.8402$). Appendix 2 details caecal and ileal enumeration data for each experimental bird of experiment 5.

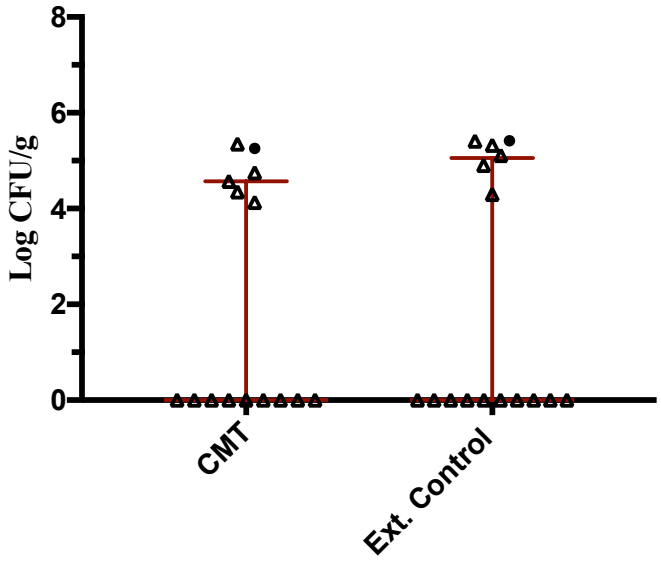


Figure 30. Levels of *C. jejuni* M1 in the ileum of broiler chickens under experimental conditions based on protocols listed for experiment 5 (CMT n=15; Ext. control n=16). Each symbol represents ileal *C. jejuni* load for an individual animal. Statistical analysis per treatment group are given as median values with associated IQR with significance determined using Mann Whitney-U analysis. All birds detected as shedding are given reported using a hollow triangle symbol, while all birds not detected as shedding *C. jejuni* are reported using a circle.

Extra-intestinal spread of C. jejuni

C. jejuni was detected in both liver and splenic tissue samples of both treatment groups of experiment 5 at point of cull. Of the 15 birds within the CMT treatment group, 5 birds (33 %) had detectable *C. jejuni* within the spleen, with 7 birds (47 %) *C. jejuni* positive within hepatic tissues. Of the 16 External control birds, a total of 3 birds (19 %) were *C. jejuni* positive within splenic tissues, with 7 birds (44 %) positive within hepatic tissues. A representation of *C. jejuni* detection within both tissue samples for all birds of experiment 5 pre- and post-enrichment is presented in Figure 31.

Treatment Group	Bird ID	*C. jejuni* positivity			
		Spleen		Liver	
		D	E	D	E
CMT Treated	2251				
	2252			■	■
	2253	■	■	■	
	2254		■	■	■
	2255*		■		
	2256				
	2257			■	■
	2258				
	2259		■		■
	2260				
	2261				
	2262*				
	2263				
	2264			■	■
	2265	■	■	■	■
External Control	1776				
	1777				
	1778				
	1779				
	1780				■
	1781				
	1782				
	1783			■	■
	1784				
	1785		■	■	■
	1786				
	1788*	■	■	■	■
	1789		■	■	
	1790				
	1791			■	■
	1792*				■

Figure 31. Detection of *C. jejuni* M1 within liver and splenic tissues of broiler chickens under experimental conditions based on protocols listed for experiment 5 (CMT n=15; Ext. control n=16). Red squares depict *C. jejuni* detection within a single sample, whereby 'D' indicates results are from direct plating of tissue homogenate and 'E' depicts results are from enriched samples.

EXPERIMENTAL TRIAL 6

Cloacal shedding

Direct challenge of all experimental animals with *C. jejuni*, as seen in experiment 6, resulted in an initial reduction in flock bacterial shedding compared to hatchery External control birds (Figure 32).

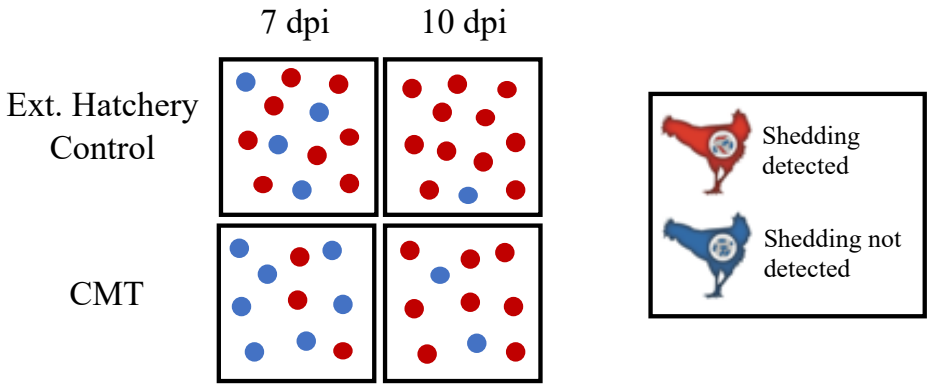


Figure 32. *C.jejuni* M1 transmission within treatment groups of experimental broiler chickens. Cloacal shedding was determined through cloacal swabbing at pre-defined time-points according to experimental protocols. Red shapes depict birds detected as shedding *C. jejuni* while blue shapes show groups with no detected bacterial shedding. CMT n=10; Ext. control n=12.

Of the External control birds 8/12 (67 %) were shedding *C. jejuni* at 7 d.p.i compared to just 3/10 (30 %) within the CMT treated group. However, by 10 d.p.i there was no apparent difference between the two treatment groups with 11/12 (92 %) External control birds shedding *C. jejuni* compared to 8/10 (80 %) CMT treated birds. A representation of *C. jejuni* shedding per treatment group for experiment 6 is provided in Figure 33.

Treatment Group	Bird ID	Cloacal swab *C. jejuni* detection					
		Pre		7		10	
		D	E	D	E	D	E
CMT Treated	1907						
	1908						
	1909						
	1910						
	1911						
	1912						
	1913						
	1917						
	1920						
	1921						
External Control	2161						
	2162						
	2163						
	2165						
	2166						
	2167						
	2170						
	2172						
	2178						
	2180						
	2182						
	2193						

Figure 33. Detection of *C. jejuni* via cloacal swabbing at time points stipulated in experiment 6 protocols. Red squares depict *C. jejuni* detection within a single swab sample, whereby 'D' indicates results are from direct plating of swab and 'E' depicts results are from enriched samples. CMT n=10; Ext. control n=12.

Caecal colonisation

Caecal samples were collected from birds of both CMT and External control treatment groups at two cull points during Experiment 6; 25 d.p.h (4 d.p.i) and 31 d.p.h (10 d.p.i) (Figure 34). Normality of distribution was not observed for the *C. jejuni* enumeration data sets of some treatment groups ($p < 0.05$) and as such, all results will be discussed and presented as group median values, including their respective interquartile range.

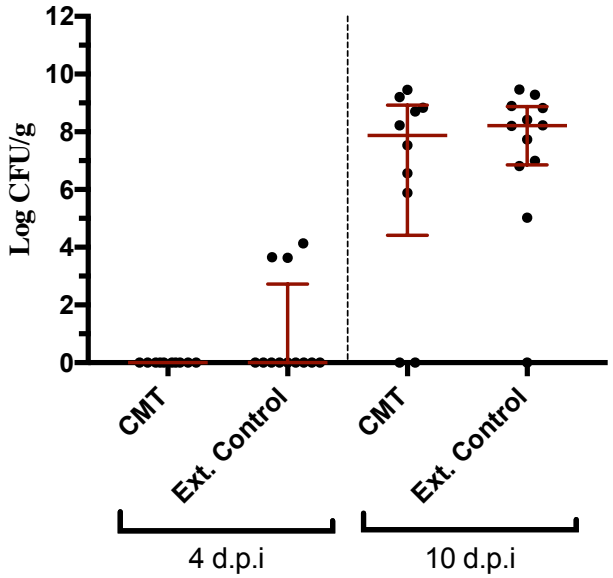


Figure 34. Levels of *C. jejuni* M1 in the caeca of broiler chickens under experimental conditions based on protocols listed for experiment 6. Each symbol represents caecal *C. jejuni* load for an individual animal. Statistical analysis treatment group are given as median values with associated IQR with significance determined using Mann Whitney-U analysis. At 4 d.p.i CMT n= 10; Ext. control n= 12, at 10 d.p.i CMT n= 10; Ext. control n= 12.

Caecal colonisation of experimental birds culled 4 d.p.i was found to be less frequent and of lower burden than that seen for birds culled 10 d.p.i. Of the 12 External control birds culled 4 d.p.i, 3 had detectable levels of *C. jejuni* within the caeca (25 %). Median *C. jejuni* colonisation of 4 d.p.i External control birds was 0.00 (IQR 2.72), with CMT treated birds showing no detectable *C. jejuni* colonisation at this time point ($p = 0.2208$). By 10 d.p.i, caecal colonisation was present within 11/12 (92 %) External control birds, with median colonisation of 8.21 (IQR 2.02). Caecal colonisation was lower in frequency within the CMT treated population, in 8/10 (80 %) of birds from this treatment group, with median colonisation of 7.88 (IQR 4.52). As with 4 d.p.i, CMT treated birds showed lower caecal *C. jejuni* burden, although this relationship was not statistically significant ($p = 0.7219$).

Ileal colonisation

As with caecal sample collected, samples of ileal content were collected from groups of birds of each treatment group at 25 d.p.h (4 d.p.i) and 31 d.p.h (10 d.p.i). Enumeration values collected for *C. jejuni* load within ileal content of birds from experiment 6 showed non-normal conformation ($p < 0.05$) and as such, these results with be discussed and presented as

medians, including their respective interquartile range. Ileal colonisation with *C. jejuni* occurred within both treatment groups of experiment 6, although colonisation was first detected at a later time-point post infection compared to that of External control birds (Figure 35).

While no birds treated with CMT were positive for *C. jejuni* within the ileal samples collected at 4 d.p.i, colonisation was evident in 2/12 External control birds (17%), although median Log_{10}CFU/g values remained at 0.00 (IQR 3.82) for this group (p = 0.4805). Interestingly, by 10 d.p.i, 7/8 CMT treated birds (88 %) were colonized by *C. jejuni* within the ileal tract, with this being 9/12 (75 %) for the External control group at the same time point. In accordance with this, CMT treated birds had a median *C. jejuni* burden of 5.13 Log_{10}CFU/g (IQR 1.68) while median External control *C. jejuni* burden was 3.91 Log_{10}CFU/g (IQR 3.80) (p = 0.0712). Appendix 2 details caecal and ileal enumeration data for each experimental bird of experiment 6.

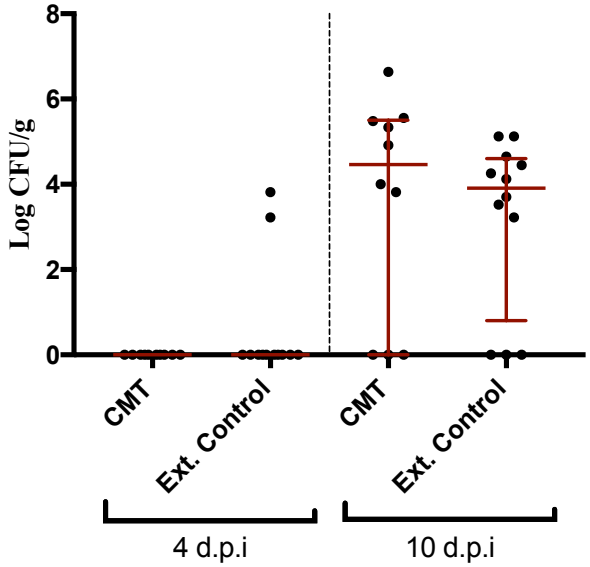


Figure 35. Levels of *C. jejuni* M1 in the ileum of broiler chickens under experimental conditions based on protocols listed for experiment 6. Each symbol represents ileal *C. jejuni* load for an individual animal. Statistical analysis per treatment group are given as median values with associated IQR with significance determined using Mann Whitney-U analysis. At 4 d.p.i CMT n= 10; Ext. control n= 12, at 10 d.p.i CMT n= 10; Ext. control n= 12.

Extra intestinal spread of C. jejuni

At post-mortem examination, no extra-intestinal spread of *C. jejuni* to either the splenic or hepatic tissues was observed for birds culled 4 d.p.i in either treatment group. By 10 d.p.i 4/12 (25 %) External control birds had *C. jejuni* colonisation of the liver tissue and 5/12 birds (42 %) of the splenic tissue. Birds treated with CMT showed no liver colonisation of *C. jejuni* at 10 d.p.i but 3/8 (38 %) had detectable *C. jejuni* within the splenic tissue. A representation of *C. jejuni* detection within both tissue samples for all birds of Experiment 6 pre- and post-enrichment is presented in Figure 36.

Treatment group	Bird ID	10 d.p.i			
		Spleen		Liver	
		D	E	D	E
CMT Treated	1907				
	1908	■	■		
	1909		■		
	1910				
	1911				
	1912	■	■		
	1913				
	1917				
	1920				
	1921				
External Control	2161				
	2162	■	■		
	2163		■		■
	2165		■		■
	2166		■		■
	2167				
	2170				
	2172				
	2178				
	2180		■		
	2182				
	2193			■	

Figure 36. Detection of *C. jejuni* M1 within liver and splenic tissues of broiler chickens under experimental conditions based on protocols listed for experiment 6 at 10 d.p.i. Red squares depict *C. jejuni* detection within a single sample, whereby 'D' indicates results are from direct plating of tissue homogenate and 'E' depicts results are from enriched samples. CMT n=10; Ext. control n=12.

HUMORAL RESPONSE

During post-mortem, serum samples were collected via cardiac puncture from all birds in experiment 4 to determine the degree of humoral response produced by the chickens to primary infection with *C. jejuni* following CMT treatment (Figure 37). Optical density readings for all data sets showed normal data distribution ($p > 0.05$) and so statistical analysis was conducted using unpaired t-tests. Birds within the External control treatment group showed highest variation in serum IgY titres compared to that of CMT treated birds. While mean serum IgY titres were highest within birds of the External control treatment group, this increase was not of statistical significance when compared against CMT treated animals ($p = 0.0983$). Conversely to that observed for serum IgY, serum IgM titres were significantly higher within birds treated with CMT at-hatch, compared to untreated birds within the External control treatment group ($p = 0.0071$).

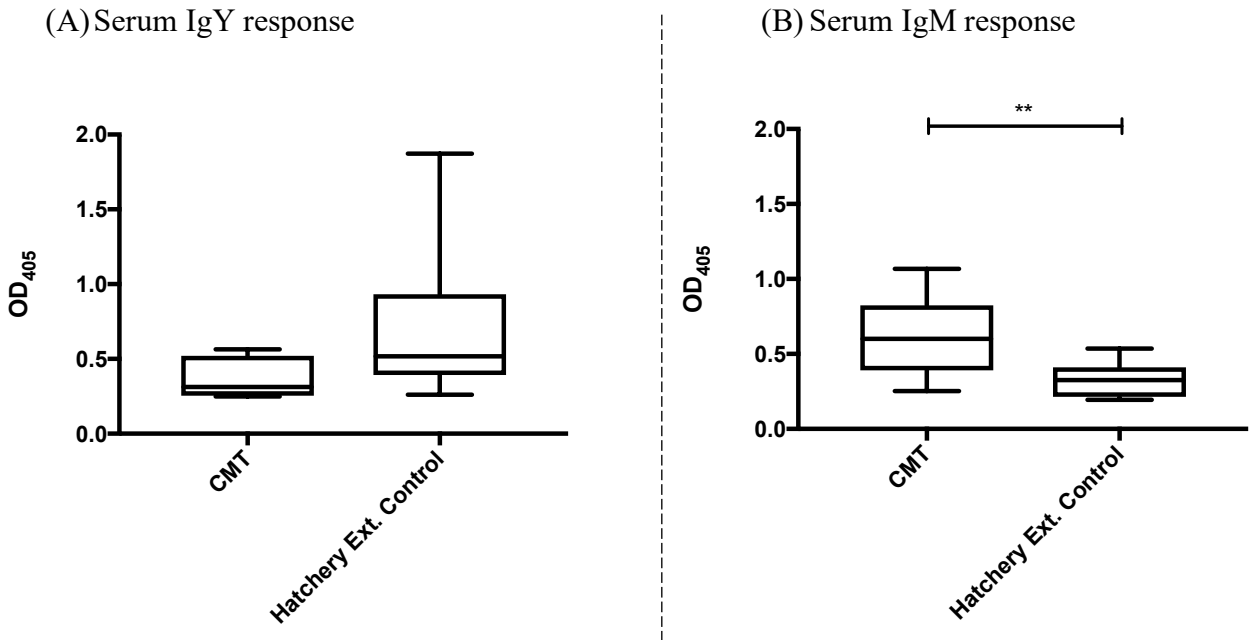


Figure 37. *Campylobacter* specific serum IgY (a) and IgM (b) responses in experimental chickens given as OD_{405} readings following ELISA protocols. All results are based on blood samples collected at *Post-mortem* from Experiment 4 at 12 d.p.i. CMT n=8; Ext. control n=12.

DISCUSSION

The results of this study show that the transplantation of a microflora preparation, derived from whole 'adult' broiler chicken microbiota directly to chicks' post-hatch, might be effective in reducing susceptibility of recipient birds to subsequent *Campylobacter* infection. In all experimental models where administration of CMT was given immediately post-hatch, subsequent within-flock transmission of *C. jejuni* was disrupted compared to chickens not given treatment. This influence was particularly evident when using a seeder model of infection whereby experiment 3 showed 19/19 hatchery External control chickens actively shedding *C. jejuni* compared to just 4/19 CMT chickens at 33 days post-hatch. Since bacterial shedding is not necessarily indicative of level of *C. jejuni* colonisation within individual birds, caecal and ileal content were collected post-mortem for bacterial enumeration. As seen with bacterial shedding, GIT colonisation of *C. jejuni* within chicks given immediate CMT post-hatch was significantly lower at both sites in comparison to that of non-treated hatchery chickens using seeder infection methodologies. CMT delivered in this way was able to prevent detectable colonisation of the ileal tract within both experiment 3 and experiment 4. Of more importance was the impact of CMT treatment on level of *C. jejuni* caecal colonisation, being its primary niche within the avian host (Humphrey et al., 2014). Impact of CMT on such colonisation was demonstrated in both experiment 3, where hatchery chicken caecal *C. jejuni* load was 6-fold higher than that of CMT chickens, and experiment 4, where CMT seemed to completely prevent detectable caecal colonisation of the CMT treatment group compared to mean colonisation of 9.06 Log_{10}CFU/g in hatchery control chickens. Comparisons can be made between these findings and those of the pioneering research from Rantala & Nurmi (1973), who demonstrated how microbiota from healthy donor chickens was able to reduce colonisation of chickens with Salmonella following experimental infection.

Poultry act as a particular novelty within farmed animal production, in that young generally receive no maternal contact post-hatch (Stanley et al., 2014). Commercial hatcheries employ strict hygiene processing protocols, with eggs being washed and fumigated following collection, a process that will erase most traces of maternal and environmental bacteria (Stanley et al., 2014). Since the development of broiler microbiome begins immediately post-hatch, it is the hatchery environment that will form the first bacterial inoculum and likely have most bearing on a chickens' microbial profile over time (Stanley et al., 2014). As such, a potentially 'humanised' chick microbiome with restricted bacterial diversity and distribution could have a detrimental impact on the ability of chickens to effectively respond to intestinal

infection and subsequent disease (Volf et al., 2016). Experimental demonstration of this understanding can be seen in work from Dicksved and colleagues (2014) using a mouse model. Although inherently resistant to *C. jejuni* infection, when artificially colonised with a humanized intestinal microbiota, experimental mice exhibited signs of intestinal inflammation commonly associated with human campylobacteriosis (Dicksved et al, 2014). Early inoculation of chicks with microbiota could offer a promising means of curtailing the detrimental effects of poultry production systems on the microbiota of broiler chickens and reduce their susceptibility to infection (Volf et al., 2016). Disease prevention through the improvement of avian gut health in this manner presents an effective method of disease prevention and control in a climate where alternatives to antibiotic use in farm animal practice are required.

Although the fundamental rationale behind FMT is similar to that of dietary supplements such as probiotics; restoration and restructuring of the intestinal microbiota to confer health benefit, the pathophysiological understanding behind probiotic therapies are considerably more developed compared to those of FMT and similarly derived Competitive Exclusion (CE) products (Chaitman et al., 2016). Unlike the temporary colonisation of the gut lumen by modern probiotic therapies, FMT infusion is more effective at the bacterial engraftment of donor microbiota, establishing both an enhanced and more durable alteration to the recipients' microbiota (Cammarota et al., 2014). It may be likely to assume that not only may the mechanisms of action described for probiotic therapies be applied for FMT treatment, these may also be exaggerated in effect (Cammarota et al., 2014).

As with probiotic therapies, one of the most postulated principles behind FMT success is its ability to competitively occupy the niche of indigenous gut microbiota. FMT action may also be characterised by promotion of host immunological defense mechanisms, mediating immune responses through pro and anti – inflammatory cytokine modulation (Isolauri et al., 2001). It is of interest that CMT treated birds had higher serum C*ampylobacter* specific IgM compared to that of untreated control birds within experiment 4. Work by Haghighi et al (2005) developed this notion of immunomodulation through use of probiotic formulations, with multiple host-microbiota mechanisms potentially underpinning important functionality of CMT against *C. jejuni* infection within our infection model.

Although FMT should be considered a true organ transplantation as oppose to simply an infusion of faecal material, donor selection for FMT is considerably easier since no direct

immunological match is necessary between donor and recipient (Cammarota et al., 2014). When considering human FMT use, multiple donor screening criteria must first be satisfied before continuation of the procedure, although optimal donor characteristics are yet to be standardized and published guidance remains limited (Chaitman et al., 2016). Of greatest importance across both human and veterinary clinical application remains the necessity to avoid transfer of disease from host to recipient. It may also be important to note that application of chicken CMT practices in countries still utilizing antimicrobials at therapeutic and sub therapeutic levels in chicken feed may have additional selection criteria to eliminate the transfer of resistance determinants between bacterial species from donor to host. Here, all control birds acting as donors of caecal material were confirmed as having *C. jejuni* negative status and were otherwise healthy at point of post-mortem.

Limitations of this work can be drawn from the preservation and preparation methods used in regard to CMT material. Within human clinical FMT use, little evidence exists for best practice to ensure preservation of donor microbiota viability, with the same being said for veterinary application. Although FMT in humans is becoming an ever-established treatment regime for infections such as *C. difficile*, processing of donor stool for transplantation is often found to vary between trials, including freezing, freeze drying and preparation across any degree of aerobic to anaerobic conditions (Papanicolas et al., 2019). Research by Papanicolas et al (2019) into specific viability implications of several preparation methods on the bacterial communities within FMT material provides insight into how the freeze-thaw preparation used in this work could influence the bacterial composition transferred by transplantation. Such work suggests that although overall transplant material viability was significantly reduced 4-fold, microbial composition of this viable microbiota was unaffected compared to anaerobically processed fresh transplantation material (Papanicolas et al., 2019). Additionally, while glycerol is commonly used as a preservative media in clinical FMT use (Cammarota et al., 2014), Dan et al (1989) postulates that this technique offers no significant benefit in faecal material quality when stored at very low temperature (- 70°C). With this being said, it continues to be a priority to ensure only short lag times are employed between transplant material collection and utilization alongside only moderate use of freeze-thaw cycles (Chu et al., 2017).

Similar to techniques employed in most clinical FMT trials communicated in American, British and European guidelines (Papanicolas et al., 2019), preparation of our caecal transplantation

material was conducted aerobically, although conscious effort was placed on minimizing the extent of this aerobic exposure. Work by Chu et al., (2017) confirms general conjecture across published literature that oxygen exposure during aerobic preparation of transplantation material will somewhat compromise viable microbiota composition within the transplanted material, although it continues to be unclear as to how these alterations may impact the potential therapeutic benefit. It may be assumed that this oxidative stress will disproportionately impair strictly anaerobic microbial families, however Chu et al., (2017) goes on to discuss particular oxygen tolerance of the strictly anaerobic *Bacteroides* genus and how this may denote insufficiency of using information on bacterial growth conditions as a predictor for the response of specific bacterial taxa to stressors within a complex community. Applying this concept to the findings from this study, it should be considered that although processed aerobically, culture of multiple strictly anaerobic bacterial taxa were confirmed through MALDI-TOF MS biotyping. As such, a definite viable community of anaerobic bacterial taxa exists within our CMT material following processing, suggesting potential for re-expansion of these taxonomic groups in the gut of transplant recipients. It should also be considered, that host immunostimulatory effects can also be induced through transfer of non-viable bacterial cells and bacterial DNA, as discussed in work by Bojanova & Bordenstein (2016). The undoubted success of CMT within our work at reducing *C. jejuni* flock transmission and subsequent colonisation provides reasoning that the transplantation methodologies employed are unlikely to negate much of the therapeutic benefit offered. Conversely, the negative influence of oxygen exposure to transplantation material is justifiably deliberated in published literature and may be an important mediator on the efficacy of CMT within our study. It would be beneficial for future work to assess the impact of oxygen exposure to CMT material during processing on subsequent efficacy against *C. jejuni* infection and implementing viability assays to ensure transplantation of a broad range of viable bacterial communities.

It is also yet to be determined how long post-administration the influence of CMT treatment might extend, and whether a protective effect against *C. jejuni* is observable at time-points more indicative of a later commercial slaughter age. Second to this, it might also be of importance to ascertain whether this effective period can be manipulated by repeated CMT dosing. Our results indicate that, although directly infected with *C. jejuni*, seeder birds were regularly not identified as shedding the bacterium until considerably after infection. To truly understand the biological basis of *C. jejuni* shedding from chickens with cloacal infection,

future work could potentially gain more useful insight by determining the exact limit of detection for *C. jejuni* using the protocols described here, while also increasing the frequency at which each individual was cloacally swabbed.

In conclusion, the novel application of CMT used here demonstrates efficacy against *C. jejuni* transmission and GIT colonisation of the broiler chicken. There remain many unanswered questions regarding clinical use of microbiota transplantation in therapeutic treatment in both human and veterinary medicine, but these data provide an interesting theory on how modification of the avian microflora at point of hatch may provide promising prophylactic therapy in reducing bacterial gastrointestinal infection.

Chapter Four: A comparison of two microflora preparations: Caecal Microbiota Transplantation and Aviguard®

INTRODUCTION

The poultry industry produces much of the global meat supply, with preference for chicken meat continuing to overshadow that seen for almost all other protein sources (FAO 2019). Poultry production is the fastest growing meat sector, contributing to over a 35% share of global meat production in 2016 (FAO, 2019). Selection pressures placed on the broiler chicken over decades of domestication have refined flock growth performance, a phenotypic trait of upmost concern for producers (Clavijo & Flórez, 2018). Although flock efficiency can be assessed using multiple parameters, the most universal measure remains the measure of food conversion ratio (FCR); a measure of growth efficiency with respect to nutrient provision (Stanley et al., 2013). Current estimates of broiler chicken FCR stand at 1.5 – 2.0, with continued industrial drive for refinement.

Crucially, FCR is dependent on the effective extraction of energy and nutrient resources from ingested feed within the gastrointestinal tract (GIT). It is this interface that presents the chicken intestinal microbiota as having potentially the most fundamental influence on broiler productivity, health and susceptibility to opportunistic disease (Clavijo & Flórez, 2018). With the economic benefit offered to industries such as the poultry industry, improvement in our understanding of the gastrointestinal (GI) microbiota, and more importantly how it can be manipulated, has been driven to the forefront of scientific research more than ever before. Where antibiotic alternatives to disease treatment and prevention have previously been available, global campaigns in reduction of such practices have meant that use of the natural microbial ecosystems to protect against production animal disease may no longer be just a viable alternative, but more one of the only tools available (Callaway et al., 2008; Mountzouris et al., 2010). Aside from directly benefiting production animal health and productivity, potential reduction in economic and labour costs further demonstrate value (Callaway et al., 2008). However, manipulation of the microbial ecosystem in this way within the poultry industry remains a relatively novel concept with limited substantiated research into how and when this microbial treatment should occur (Kabir, 2009). Although many different strategies of microbiota manipulation within the poultry industry have been suggested, it is only a selection of these that have dominated much of the ensuing research, namely probiotics and Competitive Exclusion (CE) products (Callaway et al., 2008).

As previously detailed in Chapter three, probiotics consist of a general category of dietary products delivered to enhance recipient health and performance and reduce susceptibility to

disease (Callaway et al., 2008). Although probiotic use has been widely correlated with reductions in infections such as *Salmonella enteritidis* and *Clostridium perfringens* within the poultry industry (Kizerwetter-Swida & Binek, 2005), the complexity of microbial composition offered within CE products appear to offer greater protective success against *Campylobacter* infection compared to that of more defined, simple microbial probiotic preparations (Callaway et al., 2008). Unlike CE products, probiotics used within animal production comprise of individual/mixtures of bacteria, yeasts and metabolic end products that are not species specific or even originally derived from animal origin and may not be delivered instantaneously post-hatch (Callaway et al., 2008).

Competitive exclusion products used within the poultry industry consist of a variety of anaerobic bacterial cultures usually derived from adult intestinal microbiota, applied to chicks' post-hatch, to establish early colonisation of a 'normal' protective enteric microbiota (Wagner, 2006). Such cultures can be of two forms, CE cultures with defined microbial isolates (defined CE cultures) or those whereby the microbial composition has not been completely characterised (undefined CE cultures) (Zhang et al., 2007), with both primarily intended for prophylactic use. The complexity of both host-microbe and microbe-microbe interactions occurring within the avian gut has continued to obscure the precise mechanisms by which CE microorganisms exert a protective effect (Mead, 2000). One of the most probable factors is likely to include the competition for adherence sites within the gut, a physical process evidentiarily supported by the protection of chicks from Salmonella infection only 1 hour post-treatment with a CE product (Mead, 2000; Seuna & Nurmi, 1979). It is however, unlikely that any single factor could explain the effects seen from CE treatment, and so further conceivable factors include the production of Short Chain Fatty Acids (SCFAs) (including butyric, propionic and acetic acid) by the introduced microbial groups in addition to direct competition for scarce intestinal nutrients (Zhang et al., 2007). Since first introduced, the concept of CE culture in the protection against gastroenteric disease, continued research interest has formed the basis for the manufacture of a selection of commercial CE products (Zhang et al., 2007).

Developed in Finland, BROILACT® (Orion Corporation, Espoo, Finland) was the first commercial CE product marketed for use in poultry targeting *Salmonella* infection (Nakamura et al., 2002). With use across Finland being previously reported at over 90% of newly hatched chicks in production (Nuotio et al., 2013), BROILACT® has since been used across Europe. Advertised under similar pretense to BROILACT®, several other commercial CE have been

developed and retailed, including Avifree™ and Aviguard®, MSC and Preempt™ (Nakamura et al., 2002). Developed as a CE treatment with extended shelf life compared to that of previously marketed commercial products, Aviguard® has experienced considerable commercial popularity since its launch in 1993 (Nakamura et al., 2002). Aviguard® is defined as "a natural, live intestinal microflora derived from specific-pathogen-free chickens and manufactured by fermentation" (MSD, 2009), having partially characterized microbial composition. Aviguard® has well characterised success for its capability of protecting newly hatched chicks from *Salmonella* colonisation throughout published literature (Al-Zenki et al., 2009; Ferreira et al., 2003; Nakamura et al., 2002) however little research has been performed to evaluate such protective ability against *Campylobacter* spp. Thanks to the pioneering work by Rantala & Nurmi (1973) into the fundamental concepts of CE, much of the ensuing research into CE within animals has been directed primarily toward poultry disease. However, many probiotic and CE studies within poultry systems have lacked consistency in fundamental understanding of the microbial ecology of the avian GI system and, as such, further confirmation of the effectiveness of such commercial products may be necessary with few long-term *Campylobacter* colonisation studies available (Callaway., et al., 2008; Schneitz & Hakkinen, 2016).

As discussed previously, FMT involves the transplantation of intestinal microbiota from healthy donor to recipient to introduce or restore a 'balanced' intestinal microecology (Niederwerder et al., 2018). Although more commonly discussed in reference to human clinical medicine and the treatment of CDI, interest has grown rapidly over recent years regarding veterinary application of FMT (Niederwerder et al., 2018). Peer-reviewed publications exploring therapeutic use of FMT within production animals show primary focus on swine and poultry, although much of this research is centered around clinical response to FMT treatment and less-so its potential mechanistic action (Niederwerder et al., 2018). Consequently, there are many fundamental concepts of the therapeutic action of FMT within veterinary species that are yet to be defined. Two general mechanistic principles exist for FMT, although each is largely though to be complementary to the other. One of the most commonly described is the restoration or repopulation of the intestinal microbiota improving the ability of such a microbial community to outcompete opportunistic GI pathogens for adhesion sites and metabolisable resources, similar in manner to that discussed for CE products (Mead, 2000). Supplementary to this, it is also plausible that FMT may act as an immunotherapeutic agent in the improvement of gut health and ultimate homeostasis (Chaitman et al., 2016).

Presley et al. (2010) reinforces such theory with research highlighting how a 'normal' gut microbiome within experimental mice was associated with a more cellular and responsive immune function compared to that of germ-free, pathogen-free mice.

Although conceptually similar to CMT, commercial poultry CE products could be limited in their protective efficacy against bacterial species with colonisation ecologies such as those exhibited by *C. jejuni*. *C. jejuni* is well recognized for its ability to successfully colonise poultry caecal crypts establishing a largely non-invasive persistent presence of high burden (Hermans et al., 2014). The continued latent presence of *C. jejuni* within the poultry GI tract necessitates the need for a persistently 'healthy' avian host microbiome able to prevent the opportunistic adherence and colonisation of *C. jejuni* to the enteric epithelium. As previously identified by (Khoruts, 2018), commercial bacterial formulations such as probiotics will often offer only transient modification to host microbiota compared to the long-lasting, durable alteration identified following the administration of a complete fecal microflora preparation. Although derived from chickens, production methods utilised during Aviguard® culture and fermentation are highly selective toward cultivable bacteria under specific conditions (Shang et al., 2018). A result of this may be a tendency toward reduced taxonomic richness and diversity within commercial CE products (Shang et al., 2018). Furthermore, with published research highlighting the strong influence of genetic background on intestinal microbiota composition, the use of Specific Pathogen Free (SPF) 'layer-type' chickens as a source of Aviguard® microbial flora to be used in commercial broiler production may fundamentally undermine such physiological differences between microbiota compositions (Kers et al., 2018). Maki et al. (2019) states that the vastly different production practices used between layer and broiler chicken industries makes drawing inference on any difference in breed microbiota innately complex. Although the broiler microbiome was viewed as having an increased 'simplicity' compared to that of layer type chickens, it should not be underestimated how the increased longevity of these layer chickens could influence this finding (Maki et al., 2019).

Shortcomings such as these discussed may culminate in a CE product less efficacious in establishing robust colonisation of the broiler GI tract undermining the central biological mechanism substantiating its use.

MATERIALS AND METHODS

BACTERIAL ISOLATES AND GROWTH CONDITIONS

Strain *Campylobacter jejuni* M1 was used as the infecting inoculum, prepared as previously described in Chapter 2. Serial 10 - fold dilutions of the final Mueller Hinton Broth (MHB [Lab M Ltd., Heywood, Lancashire, UK]) liquid culture were made in 1 x Maximum Recovery Diluent (MRD [Lab M Ltd, Bury, UK]) to 10^{-8} for viable colony enumeration via Miles & Misra methods (Miles & Misra 1938) and plated onto Colombia Blood Agar (CAB [Lab M Ltd., Heywood, Lancashire, UK]) supplemented with 5% defibrinated horse blood (Oxoid, Basingstoke, Hampshire, UK) as described in Chapter 2 before incubation for 48 hours at 41.5°C.

AVIGUARD® AND CMT INOCULATE PREPARATION

Aviguard® (Batch number: 1517) (Lallemand, Worcestershire, UK) was prepared as directed by manufacturers instruction for drinking water application. The entire contents of one Aviguard® packet (stated treatment sufficiency of 2000 birds) was dissolved in 1 L of deionized water, free of chlorine or disinfectant contamination. The bottle of ready-to-use Aviguard® solution was inverted routinely over 5 minutes to ensure compete dispersal of sachet contents. Once fully dissolved, the Aviguard® solution was dispersed into 2 ml aliquots and stored at - 80°C.

At point-of-use, stored 2 ml aliquots of CMT prepared to 1:20 (w/v) or Aviguard® inoculum or were warmed in a water bath at 37°C until fully thawed. Thawed aliquots of inoculum were vortexed for 1 minute to ensure thorough dispersion of contents before being delivered to recipient chicks within 1 hour of thawing. Treatment was delivered to all chicks using a 1 ml sterile syringe (Fisher Scientific, Loughborough, UK) through a sterile oral gavage (Sigma, Poole, Dorset, UK). Complete description of CMT preparation prior to aliquot dispensing and - 80°C storage can be found in Chapter 3.

EXPERIMENTAL ANIMALS

Work was conducted in accordance with United Kingdom legislation governing experimental animals under project license P999B8C93 and was approved by the University of Liverpool ethical review process prior to the award of this license. All animals held on-site were checked a minimum of twice daily to ensure individual animal health and welfare. Full description of

experimental animal housing conditions, feed and unit biosecurity measures can be found in Chapter 2, also described by Humphrey et al 2014.

EXPERIMENTAL TRIAL DESIGN

Embryonated Ross 308 hens' eggs were obtained from a commercial hatchery and transported directly to the University of Liverpool high biosecurity experimental unit. As previously described in Chapter 3, all eggs were inspected for shell quality and subsequently sterilized using a 1% (1:100 dilution) solution of Ambicide™ (PatrickPinker, Latteridge, Bristol, UK) and 1% Peracetic acid (Sigma, Poole, Dorset, UK) before transfer to a sterile incubator. All eggs were incubated for 21 days at 37.7 °C in an automatic roll incubator (Brinsea, Milton Keynes, UK). All eggs were candled at 7 days after setting to ascertain viability, with only viable eggs being retained for the remainder of the incubation period. Relative humidity was maintained at 45 – 55 % until day 18 of incubation where the humidity increased to 60 - 70 % until hatching.

A total of 87 chicks were successfully hatched following 21 days of incubation, with these being divided into three separate treatment groups;

- **CMT treated** (n = 29); received 0.1 - 0.2 ml prepared CMT inoculum within 4 hours post-hatch
- **Aviguard® treated** (n=29); received 0.1 – 0.2 ml prepared Aviguard® inoculum within 4 hours post-hatch
- **Internal control** (n=29); received 0.1 – 0.2 ml sterile 1 x Phosphate Buffered Saline (PBS [Lab M Ltd, Heywood, Lancashire, UK]) within 4 hours post-hatch

At point-of-hatch, a further 36 age matched day-old Ross 308 chicks were obtained from the same commercial hatchery, with these being divided into two groups;

- **External control** (n = 29); received 0.1 – 0.2 ml sterile 1 x PBS within 4 hours of arrival
- **Trial control** (n = 7); received no treatment

All five treatment groups were housed in separate rooms with lobbied entry and additional dedicated protective clothing and boots. For details of animal housing throughout study duration, see Chapter 2.

On day three (3 days post-hatch [d.p.h]), six birds from each group excluding the trial control group were culled via cervical dislocation. A sample of 1 – 2 g of caecal content was aseptically collected from each experimental chick by pooling entire contents of both caecal crypts. Previous work by Stanley et al (2015) has identified no statistical difference in bacterial abundance or taxonomic composition between left and right caecal crypts of the same individual. Samples were immediately snap frozen post-collection in liquid nitrogen before storage at – 80 °C and subsequent 16S rRNA gene analysis. Similarly, at 7 d.p.h a further 10 birds from each treatment group excluding the trial control group were culled and caecal samples collected in the same manner for 16S rRNA gene analysis. Further clarification on post mortem methodologies are provided in Chapter 2 and all information pertaining to the described 16S rRNA sequencing is detailed in Chapter 5.

At 14 d.p.h, all remaining birds were confirmed to have campylobacter negative status via cloacal swabbing as described in Chapter 3. At 21 d.p.h, two randomly selected birds from each group, excluding the Trial control group were orally infected with 0.2 mL 10^6 Colony Forming units/ml (CFU/ml) *C. jejuni* M1 in MHB via oral gavage. Preparation of inoculum and infection protocols were conducted according to those described in Chapter 2.

Cloacal swabs were collected from all birds at multiple time-points post infection to assess within-group *C. jejuni* shedding. Swabs were collected from all birds at 23 (2 d.p.i), 26 (5 d.p.i), 29 (8 d.p.i), 31 (10 d.p.i), 33 (12 d.p.i) and 35 (14 d.p.i) days post-hatch. Full protocols of swab bacteriological processing are provided in Chapter 3. At 35 d.p.h (14 d.p.i) all birds were culled via cervical dislocation and whole carcass weight recorded. Blood samples were collected via cardiac puncture immediately post-cull, before samples of splenic & liver tissues and caecal & ileal content were aseptically collected. Full detail on *post-mortem* aseptic sample collection alongside sample processing post-collection are provided in Chapter 2. Figure 38 provides a visual explanation of key experimental features and timelines.

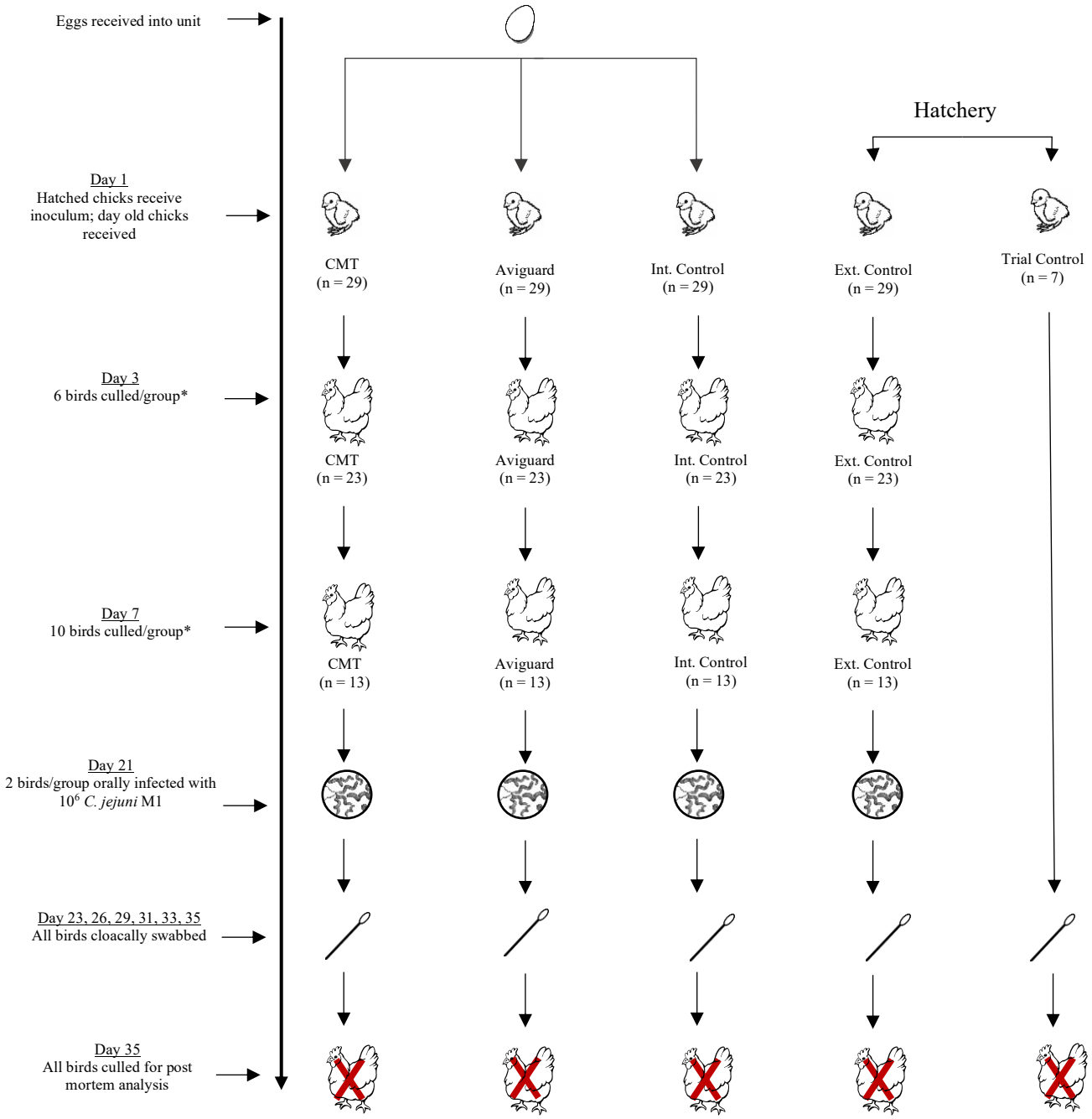


Figure 38. Key time-points associated with experimental trial 7. '*' denotes sample collected for 16S rRNA sequencing protocols described in Chapter 5.

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 7.00 for Mac OS X (GraphPad Software Inc., San Diego, USA). Prior to further statistical analysis, all data sets were assessed for normality of distribution using D'Agostino & Pearson normality testing. Data sets showing non-normal distribution ($p < 0.05$) were further assessed for statistical significance using an Unpaired t-test and described using data mean and standard deviation, with significance set as $p < 0.05$. Pairwise treatment group comparisons of non-normally distributed data sets were conducted using a Mann Whitney-U test and described using data median and interquartile range (IQR) with statistical significance set at $p < 0.05$.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Post-mortem blood samples were aseptically collected via cardiac puncture from all birds at 35 d.p.h (14 d.p.i). All blood samples were centrifuged at 13000 x *g* for 5 minutes before serum collection and storage at - 20°C for subsequent ELISA analysis. All samples were assessed for *Campylobacter* specific serum immunoglobulin-G (IgY) and IgM levels through ELISA protocols described in Chapter 2. Using the same protocols, specific serum IgA immunoglobulin levels were also assessed using alkaline phosphatase conjugated to goat anti-chicken IgA (1:2000).

IN-VITRO STUDIES

All cell culture protocols were performed under a laminar flow hood using appropriate aseptic tissue culture techniques. 8E11 cells were used in all experiments, with this cell line being derived from chicken small intestinal epithelial cells. The cell line was maintained as continual laboratory cell stock. Through pre-exposure of this avian intestinal epithelial cell line to different treatment conditions, we were able to ascertain whether treatment could induce cell specific changes that protect against bacterial invasion.

Revival of 8E11 cells

Seed stock of 8E11 cells were cryopreserved in sterile cryogenic storage vials (STARLAB, Milton Keynes, UK) in liquid nitrogen until use. Storage of cells was in complete medium in the presence of the cryoprotective agent dimethylsulfoxide (DMSO) (Sigma, Poole, Dorset, UK).
The adherent 8E11 cell line was cultured in Gibco® Dulbecco's Modified Eagles Medium: Nutrient Mixture F-12 (DMEM/F-12) (ThermoFisher Scientific, Loughborough, UK)

supplemented with 10 % (v/v) fetal bovine serum (Sigma-Aldrich, Poole, UK), 2 % Penicillin/Streptomycin solution (Sigma-Aldrich, Poole, UK) and 2 % L-glutamine solution (Sigma-Aldrich, Poole, UK). Upon revival, 8E11 cells were thawed for one minute in a water-bath set to 37°C, gently agitating the vial during this period. Once completely thawed, the vial contents were transferred directly to 15 ml of complete culture medium, pre-warmed to 37°C, in a T75 tissue culture flask (STARLAB, Milton Keynes, UK) before incubation at 37°C, 5 % CO_2 for 24 hours. After this initial incubation, cells were checked for flask adherence, signs of contamination and general cell layer dispersion before the conditioned growth media was aspirated and replaced with 20 ml of fresh complete growth medium. Cells were routinely sub-cultured every 3 – 4 days to preserve cell viability.

Cell subculture

All media was pre-warmed before use in a water bath set at 37°C. Conditioned culture media was first aspirated and disposed of in a 1 % Virkon® solution. The adhered cells were rinsed 2 times using a wash of 5 – 10 ml of 1 x PBS followed by a rinse using 2 ml 1 x PBS-EDTA-Trypsin (Sigma-Aldrich, Poole, UK) solution. Another 2 ml 1 x PBS-EDTA-Trypsin was added before incubation at 37°C, 5 % CO_2 for 5 – 10 minutes or until the cells visibly detach from the flask surface. During this time, flasks were routinely agitated to aid in cell detachment. When completely detached, 18 ml of complete growth medium was added to inhibit Trypsin action and pipetted gently to prevent cell clumping. Three sterile T75 tissue culture flasks were seeded with 4 ml of cell solution, with the original flask now containing 6 ml of cell solution. All four flasks were diluted to a final volume of 20 ml by the addition of pre-warmed complete culture medium before incubation at 37°C, 5 % CO_2.

Cell plating

Three days after cell seeding, as described above, the three culture flasks seeded with 4 mL of culture solution were assessed under a microscope for visible confluency (ideally between 70 – 90 %). These flasks were subsequently trypsinised as described above, before 8 mL of complete media added to inhibit the trypsin and gently pipetted to prevent cell clumping. Cell solution from these three flasks was pooled into a separate sterile T75 flask, with this flask being gently swirled to ensure all cells were evenly distributed, before 50 μl aliquoted into a 1.5 ml microcentrifuge tube containing 50 μl Trypan Blue Stain (ThermoFisher Scientific, Loughborough, UK) creating a 1:1 solution. 10 μl of cell suspension plus Trypan Blue was

pipetted onto a haemocytometer in both chambers underneath the glass coverslip, allowing the cell suspension to be drawn over the haemocytometer by capillary action.

Haemocytometers are a quick and commonly used tool in the measurement of cell viability and number within a given sample. The number of cells suspended in a given area on the haemocytometer matrix, observed under magnification are counted to give an estimate on total cell count. Using a 10 X objective, a microscope was used to focus on the haemocytometer grid (Figure 39) allowing all viable cells to be counted. All cells stained blue (those taking up Trypan Blue) are non-viable and were excluded from any count, while colorless cells represent live, viable cells that can be included in the cell count. When counting, cells were only counted if they were set within a central grid square or on the right-hand or bottom boundary of the highlighted corner square shown in Figure 39. The same process was continued for the remaining three large corner grid squares.

Once counted, an estimate of total cell count within the pooled cell solution can be calculated using the following formula;

$$Total\ cells\ per\ mL = \frac{Total\ number\ of\ cells\ counted\ X\ dilution\ factor\ X\ \frac{10{,}000\ cells}{mL}}{number\ of\ haemocytometer\ squares\ counted}$$

The cell concentration was then multiplied by the original pooled cell solution volume to get the total number of cells estimated within our total sample volume. This cell solution was then diluted to 1 x 10^5/ml with pre-warmed complete medium and seeded into 24-well tissue culture plates (STARLAB, Milton Keynes, UK) at a volume of 1 ml per well. Cell plates were incubated at 37°C, 5 % CO_2 for 2 - 3 days or until fully confluent.

Once fully confluent, conditioned cell medium was aspirated from each well. 1 ml of 1 x PBS was added to each well and subsequently aspirated, with this forming 1 rinse of each well. A further 2 rinses were complete for all wells before the addition of 1 ml antibiotic-free medium (Nutrient Mixture F-12 (DMEM/F-12) (ThermoFisher Scientific, Loughborough, UK) supplemented with 10 % (v/v) fetal bovine serum (Sigma-Aldrich, Poole, UK) and 2 % L-

glutamine solution (Sigma-Aldrich, Poole, UK) to each well. Plates were incubated for 24 hours at 37°C in a 5 % CO_2 incubator.

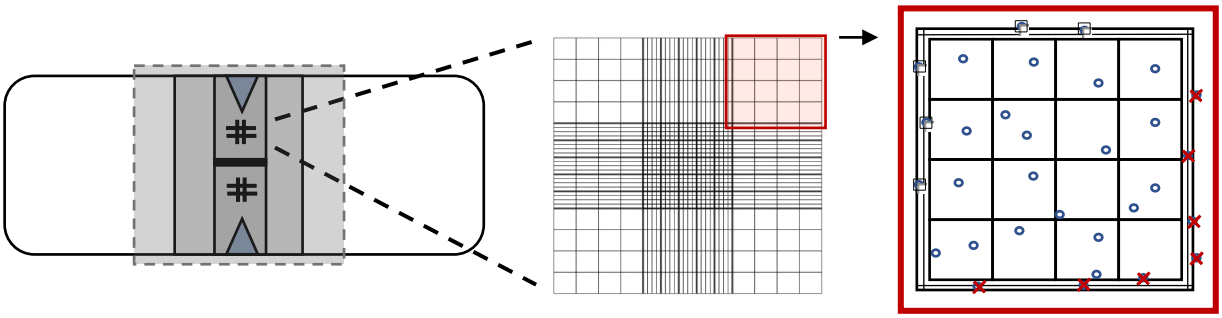


Figure 39. Haemocytometer diagram indicating one block of 16 squares used for cell counting

BACTERIAL ISOLATES AND GROWTH CONDITIONS

Two *C. jejuni* isolates (*C. jejuni* M1 and *C. jejuni* 13126) and one *S.* Typhimurium isolate (*Salmonella enterica subsp. enterica* serovar Typhimurium str. 4/74) were included in gentamicin protection assay protocols. *C. jejuni* M1 was the bacterial isolate used in all *in vivo* infection models throughout this work and representing a commonly isolated field strain. *C. jejuni* 13126 also represents a commonly isolated field strain, but more importantly has been reported by Humphrey et al. (2014) as being a rapidly invasive phenotype in both human epithelial cell lines (Caco-2) and *Galleria* insect models compared to other *C. jejuni* isolates. With such differences in *C. jejuni* infection biology between exhibited between strains, assessing the protective capacity of CMT and Aviguard® against these different invasive capabilities may have important implications in utilizing these products as control strategies.

For *C. jejuni* culture, stock strains stored at - 80°C were grown on CAB agar supplemented with 5% defibrinated horse blood (Oxoid, Basingstoke, Hampshire, UK) at 41.5°C for 48 hours under microaerobic conditions (80 % N_2, 12 % CO_2, 5 % O_2 and 3 % H_2). A single colony from the grown bacterial culture was selected and used to inoculate 10 ml of MHB in a sterile 30 ml universal tube. The 10 ml working liquid culture was grown for 24 hours under microaerobic conditions at 41.5°C with a loosely capped lid. After overnight incubation, the liquid culture was vortexed and 2 ml transferred to a sterile cuvette. Optical density (OD) was measured using a spectrophotometer (Cecil CE2040, Cambridge, UK) and adjusted using MHB to an OD_{600} of 0.1 – 0.3, corresponding to 1 x 10^8 CFU/ml of the specific *C. jejuni* strain. A further dilution of 1:200 (v/v) of the adjusted liquid culture into 20 ml MHB was made. Serial 10 - fold

dilutions of the final liquid culture were made in 1 x MRD to 10^{-8} and plated onto CAB (Lab M Ltd., Heywood, Lancashire, UK) supplemented with 5% defibrinated horse blood (Oxoid, Basingstoke, Hampshire, UK) via the previously described Miles & Misra method to allow for enumeration (Miles & Misra, 1938).

In addition to the described *C. jejuni* bacterial challenge strains selected, *Salmonella* Typhimurium 4/74 was also used as a challenge organism. Strain 4/74 was originally isolated from a calf suffering salmonellosis and has well documented virulence in cattle, pigs, chicken and mice (Chaudhuri et al., 2013). *S.* Typhimurium ST4/74 was similarly grown from - 80°C stored stock strains onto CAB agar (Lab M Ltd., Heywood, Lancashire, UK) supplemented with 5% defibrinated horse blood (Oxoid, Basingstoke, Hampshire, UK) before incubation for 24 hours at 37°C. A single bacterial culture was selected and used to inoculate 10 ml of Luria-Bertani (LB) broth (Lab M Ltd., Heywood, Lancashire, UK) in a sterile 30 ml universal tube with loosely capped lid. The 10 ml working liquid culture was grown for 24 hours at 37°C in an orbital shaking incubator at 150 rpm. After overnight incubation, the liquid culture was vortexed, and 2 ml placed in a cuvette for OD analysis as previously described for *C. jejuni* strains. A further dilution of 1:200 (v/v) of the adjusted liquid culture into 20 ml LB broth was made. Serial dilutions of the final liquid culture were made in 1 x PBS to 10^{-8} and plated onto LB agar (Lab M Ltd., Heywood, Lancashire, UK) via the previously described Miles & Misra method to allow for enumeration (Miles & Misra, 1938). Non-infected control cells were inoculated with sterile broth in accordance with that used for all other cells, i.e. MH Broth for *Campylobacter* protocols or LB Broth for *Salmonell*a protocols. Such methodologies allowed us to minimise the potential impact of culture media on cell response.

AVIGUARD® AND CMT INOCULATE PREPARATION

CMT (1:20 [w/v]) material were obtained as described previously in Chapter 3. Aviguard® material was dispersed in 1L of deionized water as previously described in Chapter 3 before being incubated at 37°C in a sealed container for 24-hours. To prepare the filtrate material for each inoculum, 10 ml of each individual inoculum was passed through a sterile 0.22 µm Millex-GP syringe filter (Sigma-Aldrich, Poole, UK) to remove bacteria suspended in either solution. This filtered supernatant was stored at - 80°C until use.

GENTAMICIN PROTECTION ASSAY (GPA)

To assess the protective ability of CMT and Aviguard® filtrate in reducing the invasiveness of *C. jejuni* and *S.* Typhimurium into epithelial cells, a gentamicin protection assay was performed. This widely used enumeration protocol was used as an assessment of the Colony Forming Units (CFU) of bacteria infecting cultured avian intestinal epithelial cells after killing extracellular, non-invading bacteria with gentamicin treatment. Following the overnight incubation of 8E11 cells in antibiotic free medium, cells were inoculated with 100 μl/well of either control inoculum, CMT filtrate or Aviguard® filtrate. Control inoculum consisted of syringe filtered 1 x PBS. All plates were incubated for 2 hours at 37°C, 5 % CO_2. Immediately following incubation, cells were infected with 100 μl/well of adjusted *C. jejuni* or S. *Ty*phimurium culture, leaving two wells per treatment group, per infection strain, non-infected. The infection was allowed to proceed for 4 hours at 37°C, 5 % CO_2.

After 4 hours of infection, the host-pathogen mixture was washed three times with 1 x PBS to remove excess extracellular bacteria alongside any non-adherent 8E11 cells. Wells were overlain with 1 ml/well DMEM F12 media supplemented with 100 μg/ml of Gentamicin sulphate (Sigma-Aldrich, Poole, UK) and incubated for 1 hour at 37 °C, 5 % CO_2 to kill the remaining extracellular bacteria that did not invade the adhered tissue cells. Cells were washed twice with 1 x PBS to remove any killed extracellular bacteria. 8E11 cells were lysed by the addition of 1 ml/well of 1 x PBS containing 0.5 % Triton-X100 (Sigma-Aldrich, Poole, UK) before incubation for 5 - 10 minutes at room temperature, with cell suspension being pipetted up and down vigorously to ensure maximal lysis. For *C. jejuni*, serial dilutions of cell supernatant to 10^{-8} were made in MRD and plated onto CAB agar (Lab M Ltd., Heywood, Lancashire, UK) supplemented with 5% defibrinated horse blood (Oxoid, Basingstoke, Hampshire, UK) according to methods previously described by Miles & Misra (Miles & Misra, 1938) and incubated for 48 hours at 41.5°C in microaerophilic conditions (80 % N_2, 12 % CO_2, 5 % O_2 and 3 % H_2). All agar plates were subsequently enumerated for internalised *C. jejuni*. For *S.* Typhimurium ST4/74, serial dilutions of cell supernatant to 10^{-8} were made in PBS and plated onto LB agar according to methods previously described by Miles & Misra (Miles & Misra, 1938) before being incubated for 24 hours at 37°C to allow for enumeration of internalized *S.* Typhimurium. A visual representation showing the key stages of the adapted gentamicin protection assay performed can be seen in Figure 40.

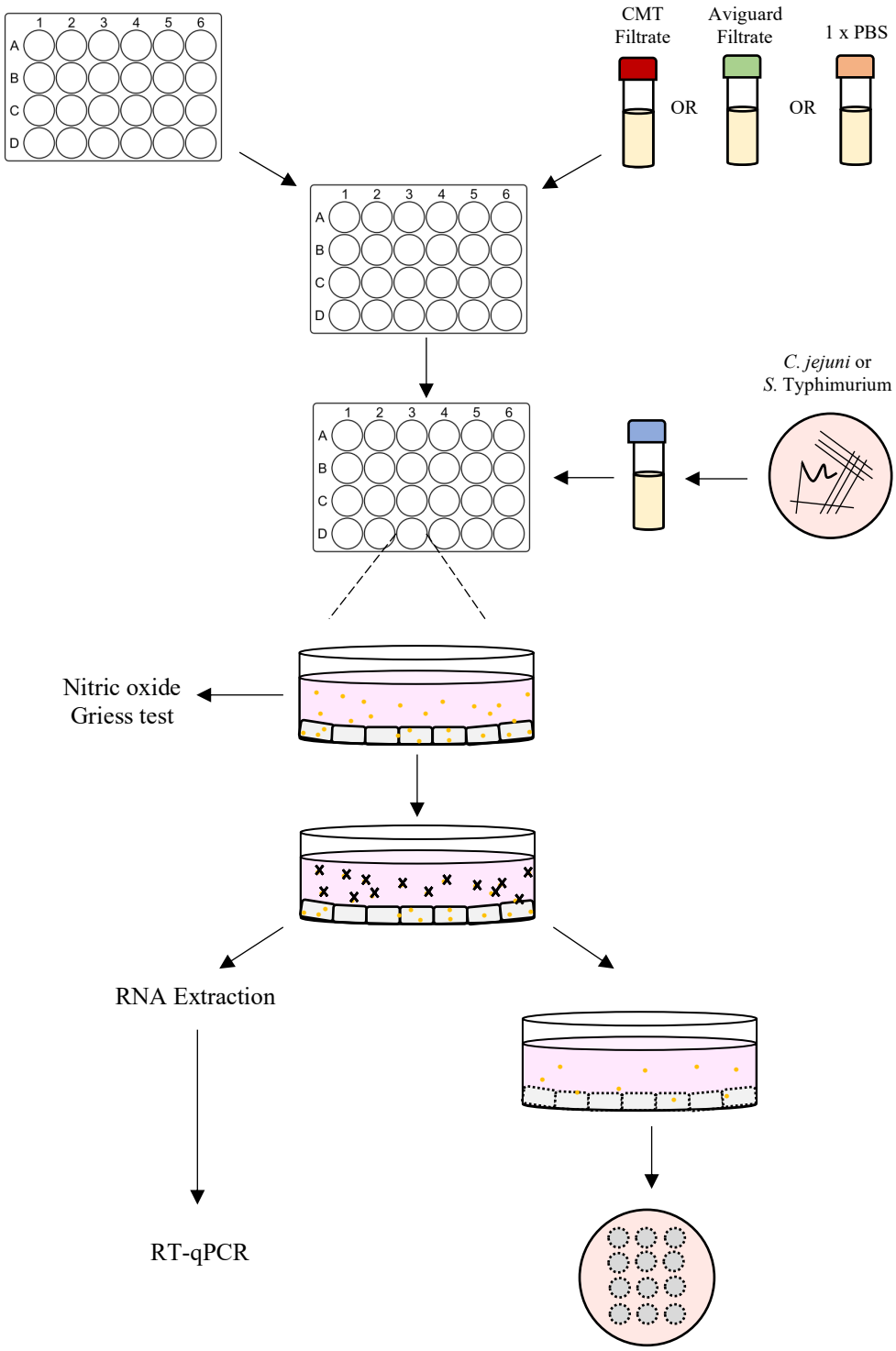


Figure 40. Representation of key stages in the adapted gentamicin protection assay

STATISTICAL ANALYSIS

The average CFU of internalized bacteria (bacteria surviving the GPA) for the most appropriate dilution series was enumerated for each replicate (each well). The percentage of internalized bacteria for each replicate was determined by dividing the CFU/ml recovered from that well by the CFU/ml of inoculum used to infect cells during GPA protocols.

Statistical analysis was performed using GraphPad Prism 7.00 for Mac OS X (GraphPad Software Inc., San Diego, USA). Prior to further statistical analysis, all data sets were assessed for normality of distribution using D'Agostino & Pearson normality testing. Pairwise treatment group comparisons of normally distributed data sets were conducted using an Unpaired t-test and described using data mean and standard deviation. Pairwise treatment group comparisons of non-normally distributed data sets were conducted using a Mann Whitney-U test and described using data median and IQR. All test statistics whereby $p < 0.05$ were considered as statistically significant.

NITRIC OXIDE PRODUCTION ASSAY

Intestinal epithelial cells are known to produce Nitric Oxide (NO) from their apical surface as a form of host defense from foreign pathogen. Nitrite is a stable metabolite of NO and is commonly measured within samples using the Griess Assay (Bryan & Grisham, 2007). Here, 8E11 cells were cultivated as previously described and seeded into 24 - well plates (STARLAB, Milton Keynes, UK) at a cell concentration of 1×10^5 cells per well. Once fully confluent, conditioned cell medium was aspirated from each well. 1 ml of 1 x PBS was added to each well and subsequently aspirated, with this forming 1 rinse of each well. A further 2 rinses were complete for all wells before the addition of 1 ml antibiotic-free medium containing no phenol red (Nutrient Mixture F-12 (DMEM/F-12, no phenol red) (ThermoFisher Scientific, Loughborough, UK) supplemented with 10 % (v/v) fetal bovine serum (Sigma-Aldrich, Poole, UK) and 2 % L-glutamine solution (Sigma-Aldrich, Poole, UK) to each well. Plates were incubated for 24 hours at 37°C in a 5 % CO_2 incubator.

Following the overnight incubation of 8E11 cells in antibiotic free medium, cells were inoculated with 100 μl/well of either control inoculum, CMT filtrate or Aviguard® filtrate. Control inoculum consisted of syringe filtered 1 x PBS. All plates were incubated for 2 - hours at 37°C, 5 % CO_2. Immediately following incubation, cells were infected with 100 μl/well of adjusted *C. jejuni, S.* Typhimurium culture. A total of 12 replicate wells were created per

treatment, per infecting strain leaving a further 5 replicate wells without treatment or infection. Two time points were of investigation and so the infection was allowed to proceed for either 4 hours or 24 - hours at 37°C, 5 % CO_2. Immediately post-incubation, 150 µl of culture supernatant from each well was transferred to the wells of a sterile flat-bottomed 90 - well plate in duplicate (STARLAB, Milton Keynes, UK) and mixed with 130 µl of deionized water.

A sufficient volume of Griess reagent was prepared by mixing equal volumes of N-(1-naphthyl) ethylenediamine and sulfanilic acid (ThermoFisher Scientific, Loughborough, UK) before 20 µl of the prepared reagent was added to each well. After 30 minutes of incubation, absorbance was measured relative to nitrite standard solutions of known concentration in a spectrophotometric microplate reader at 550 nm. A well containing only cell culture media, deionized water and Griess reagent in the quantities previously stated was used as a reference. When analysing results, the OD_{550} absorbance reading for the reference well was deducted from all other well absorbance readings. A standard curve was created of nitrate concentration (x-axis) against absorbance (y-axis) using the absorbance readings from the nitrite standard solutions prepared and sample nitrite concentrations interpolated.

RNA EXTRACTION AND RT-qPCR

Quantitative reverse transcription PCR (RT-qPCR) was performed to assess the expression of central mediators produced by our cultured cell line during the artificial 'host'-pathogen interaction orchestrated during the GPA. As shown in Figure 40, RNA was harvested from cells designated for RT-qPCR at the GPA stage immediately post 1-hour incubation with DMEM/Gentamicin. The gentamicin containing media was aspirated from each well, and all wells washed twice with 1 x PBS as described previously. 200 µl of 1 x PBS-EDTA-Trypsin was added to each well and incubated at 37°C for 2 minutes, or until cells have visibly detached. An overlay of 500 µl of non-supplemented DMEM/F12 cell media was added to each well to quench the trypsin before the well contents was aspirated and dispensed into individually labelled 2 ml sterile eppendorf tubes. Samples were centrifuged at 300 x *g* for 5 minutes until cells formed a visible pellet at the base of the eppendorf tube. The supernatant was aspirated and the pellet containing total sample RNA dispersed in 20 µl RNase free water before storage at – 80°C for no longer than 3 days.

Total RNA was isolated from cell pellets using a Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) as described by the manufacturer according to 'Purification of Total RNA from Animal Cells using Spin Technology' protocols. Briefly, an appropriate volume of buffer RLT supplemented with 1% (v/v) β - mercaptoethanol (Sigma-Aldrich, Poole, UK) was added the cell pellet to disrupt the cells. The disrupted cell lysate was vortexed for 1 minute at full speed before being passed through a blunt 20-gauge needle using a sterile RNase free syringe. One volume of 70 % ethanol was added to the homogenized cell lysate and mixed by pipetting before transferring 700 µl of sample to a supplied RNeasy spin column. Samples were centrifuged for 15 seconds at > 8000 x *g*, flow through discarded and collection tube replaced. 700 µl of Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 seconds at > 8000 x *g* before flow through was discarded and collection tube replaced. 500 µl of Buffer RPE (diluted 1:4 from concentrate using 100 % Ethanol to obtain working solution) was added to the RNeasy spin column and centrifuged as above for 15 seconds before discarding throughflow and replacing collection tube. This step was repeated once, using an extended centrifugation of 2 minutes. The RNeasy spin column was placed in a new collection tube and centrifuged at 18,000 x *g* for 1 minute. The RNeasy spin column was placed into a sterile 1.5 mL eppendorf and 30 µl of RNase-free water added directly to the spin column membrane. The sample was centrifuged at 9,000 x *g* for 1 minute to elute RNA. RNA samples were stored at – 80°C for no longer than 2 weeks.

All extracted samples were assessed for RNA quality and concentration using a Nanodrop (ND-1000) spectrophotometer before being diluted 1:4 (v/v) to attain a final concentration of 20 ng/µl using RNase-free water supplied. All RT-qPCR analysis was conducted according to protocols discussed in Chapter 2. Reference and target gene primer and probe (if applicable) sequences are provide in Chapter 2. Expression analysis was conducted for the following target genes - IL-1β, IL-6, IL-10, TGFβ_4, CXCLi1, CXCLi2, MUC2. SYBR Green RT-qPCR techniques were also used for assessment of the antimicrobial peptide (AMP) sequence for avian beta-defensin 9 (AvBD9) using the cycling conditions listed in Chapter 2 and the following primer sequences; F: ACCGTCAGGCATCTTCACAG R: CCATTTGCAGCATTTCAGC (Hong et al., 2012). AvBD9 has been identified as having bactericidal properties against a number of bacteria associated with commercial poultry, including *Salmonella* (Sunkara et al., 2011).Both AvBD1 and AvBD6 have also previously been associated with upregulation in broiler ileal and caecal tissue in response to *Campylobacter* infection, however primers designed for both genes

proved ineffective for our tissue samples and so have not been included in the results (Garcia et al., 2018).

RESULTS

IN VIVO EXPERIMENTAL TRIAL RESULTS

Cloacal shedding

Between 23 d.p.h (2 d.p.i) and 35 d.p.h (14 d.p.i), cloacal swabs were used to determine the dynamics of *C. jejuni* infection within each experimental flock. Birds were shedding *C. jejuni* as early as 2 d.p.i in Aviguard®, External and Internal control groups, with these positive birds all being seeder birds directly infected with *C. jejuni* M1. By 10 d.p.i all birds in both the Aviguard® treated and Internal control groups tested positive for *C. jejuni* shedding, compared to 6/13 (46%) for External control birds and 0/13 (0%) for CMT treated birds. The percentage of birds shedding *C. jejuni* in the External control group reached maximum at 12 d.p.i with 10/13 (77 %) detected as *C. jejuni* positive. At 14 d.p.i, detected birds shedding *C. jejuni* was 1/13 (8 %) for CMT treated, 13/13 (100%) for Aviguard® treated, 9/13 (69%) for External control and 13/13 (100%) for Internal control experimental birds. All birds in the non-infected trial control group showed no shedding of *C. jejuni* throughout the course of the experimental trial phase. A representation of *C. jejuni* shedding positivity per group is provided in Figure 41, with swab results for uniquely identified experimental animals provided in Figure 44.

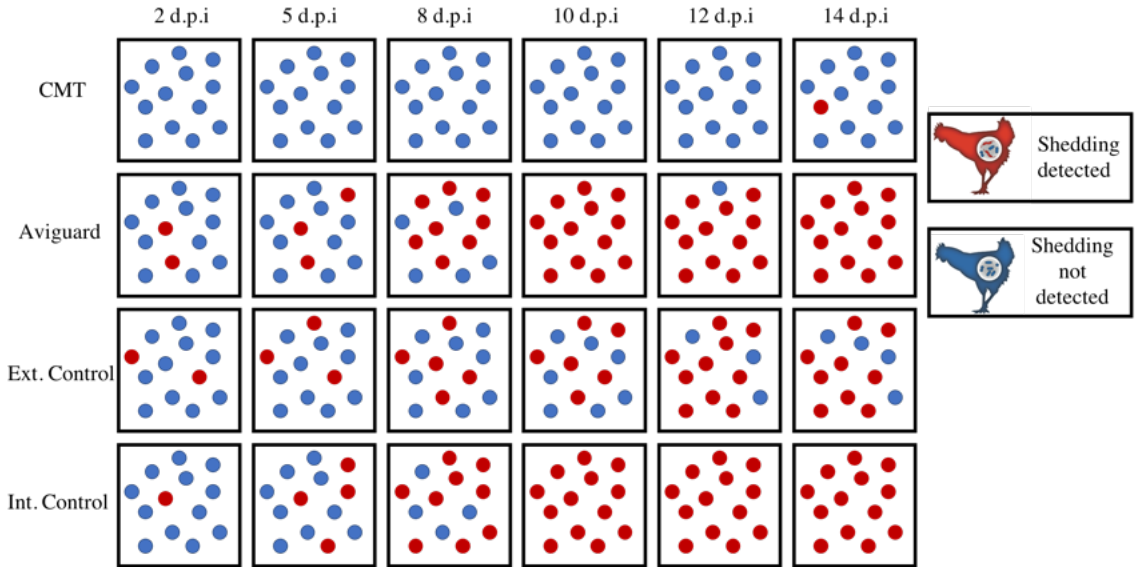


Figure 41. *C. jejuni* M1 transmission within groups of experimental broiler chickens. Cloacal shedding was determined through cloacal swabbing at pre-defined time-points according to experimental protocols. Red shapes depict birds detected as shedding *C. jejuni* while blue shapes show groups with no detected bacterial shedding. For all treatment groups, n=13.

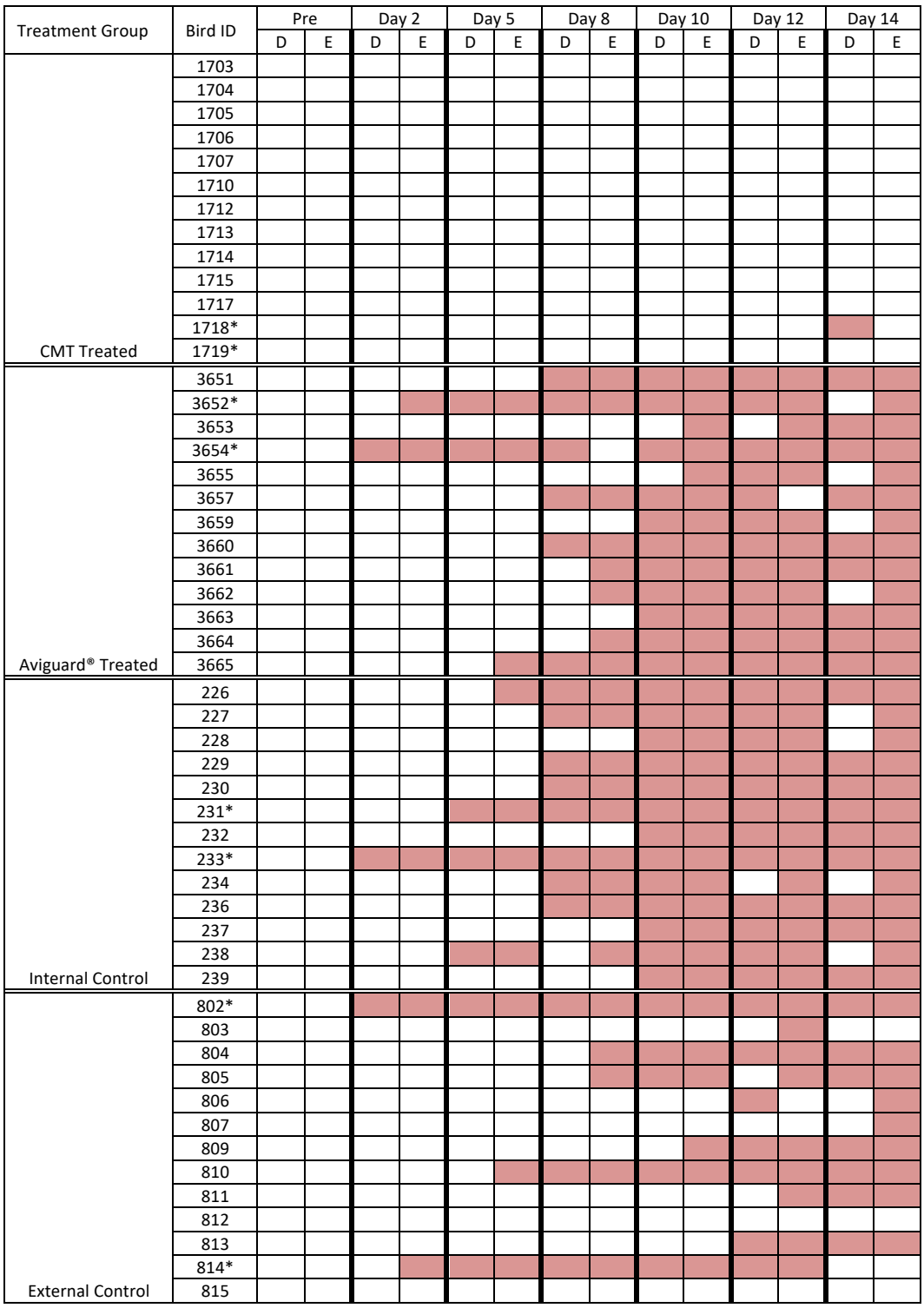


Figure 42. Detection of *C. jejuni* via cloacal swabbing at time-points stipulated in experiment 7 protocols. Red squares depict *C. jejuni* detection within a single swab sample, whereby 'D' indicates results are from direct plating of swabs and 'E' depicts results are from enriched swab samples. All birds showing '*' were directly infected as seeder birds. For all treatment groups, n=13.

CAECAL COLONISATION

Caecal content was taken from each bird at post-mortem (35 d.p.h) 14 d.p.i. All birds in the non-infected trial control group were negative for *C. jejuni* colonisation and will not be discussed further in any detail. Caecal *C. jejuni* colonisation was present in all experimental birds of both the Aviguard® treated and the Internal control experimental groups. Of the birds acting as External controls, 12/13 (92%) showed *C. jejuni* colonisation of the caecal content, with this being only 1/13 (8 %) in the CMT treated group. All birds shown to be shedding *C. jejuni* through cloacal swabbing analysis were positive for *C. jejuni* within the caecal content. A graphical representation of Log_{10} CFU/gram *C. jejuni* within the caecal content of birds in each treatment group is provided in Figure 45.

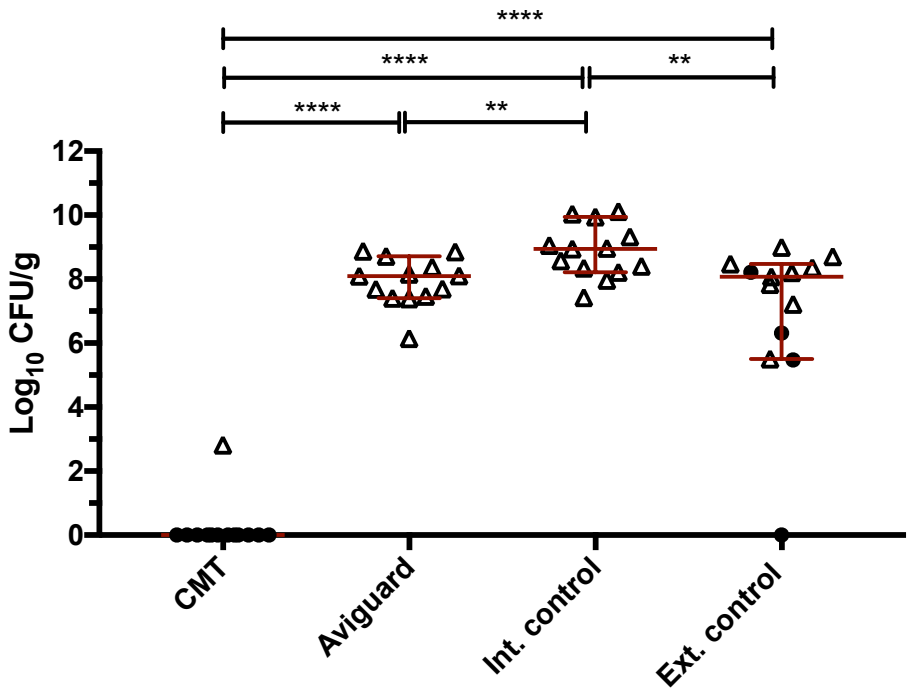


Figure 43. Levels of *C. jejuni* in the caeca of broiler chickens under experimental conditions based on the protocol listed for experiment 7. Each symbol represents caecal *C. jejuni* load for an individual animal. Statistical analysis per treatment group are given as median values with associated IQR. Significance was determined using Mann Whitney-U analysis with levels of significance given as **p < 0.01, ****p < 0.0001. All birds detected as shedding are given reported using a hollow triangle symbol, while all birds not detected as shedding *C. jejuni* are reported using a circle. For all treatment groups, n=13.

Enumeration data collected on *C. jejuni* colonisation of the caeca was not normally distributed for all treated groups and so the results are being discussed and presented as medians,

including their respective interquartile range, given as lower (Q1) and upper (Q3) quartile values. Caecal *C. jejuni* colonisation showed the Internal control experimental population having the bacterial load, with a median log_{10} CFU/gram value of 8.94; compared to that of Aviguard® (8.10), External (8.07) and CMT (0.00) treatment groups. *C. jejuni* colonisation was significantly lower in the caecal content of both Aviguard® (p = 0.0051) and External control (p = 0.0051) groups compared to that of the Internal control group. There was no significant difference in levels of *C. jejuni* colonisation of the caeca between birds given Aviguard® treatment and those acting as External controls (p = 0.6139). CMT treated birds had significantly lower caecal *C. jejuni* load compared to all three other treatment populations (p < 0.0001). Details of statistical parameters and group comparison significance values are provided in Table 14.

Table 14. Statistical parameters and treatment group comparison significance values determined for Log_{10}CFU/g *C. jejuni* caecal colonisation

Treatment group	*C. Jejuni* Load (Log_{10} CFU/g)			Data normality (p-value)	Statistical test used	Group comparison	p-value
	Median	Quartiles					
		Q1	Q3				
CMT	0.00	0.00	0.00	<0.0001	Mann Whitney-U	Aviguard® Internal Control External Control	<0.0001 <0.0001 <0.0001
Aviguard®	8.10	7.43	8.54	0.1833	Mann Whitney-U	Internal Control External Control	0.0051 0.6139
Internal control	8.94	8.28	9.63	0.8589	Mann Whitney-U	External Control	0.0051
External control	8.07	5.91	8.41	<0.0001	N/A	N/A	N/A

ILEAL COLONISATION

As described for caecal content, samples of ileal content were collected from each bird at post mortem 14 d.p.i. All birds in the non-infected trial control group were negative for *C. jejuni* colonisation and will not be discussed further in any detail. In all treatment groups, detectable presence of *C. jejuni* was less frequently observed within ileal content compared to that of the caeca. Of the 13 experimental birds in the CMT treatment group, all showed no detectable *C. jejuni* colonisation of the ileum. In all other treatment groups *C. jejuni* was detected in ileal content, with 10/13 (77%) Aviguard® treated, 9/13 (69%) Internal control and 8/13 (62%) External control birds positive for *C. jejuni* to some degree. A graphical representation of Log_{10}

CFU/gram *C. jejuni* within the ileal content of birds in each treatment group is provided in Figure 44.

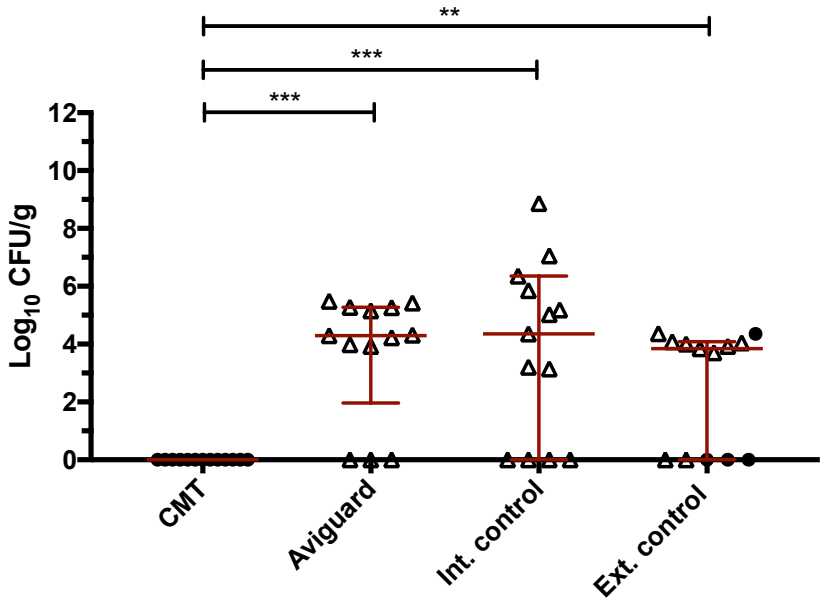


Figure 44. Levels of *C. jejuni* in the ileum of broiler chickens under experimental conditions based on the protocol listed for experiment 7. Each symbol represents ileal *C. jejuni* load for an individual animal. Statistical analysis per treatment group are given as median values with associated IQR. Significance was determined using Mann Whitney-U analysis with levels of significance given as **p < 0.01, ***p < 0.001. All birds detected as shedding are given reported using a hollow triangle symbol, while all birds not detected as shedding *C. jejuni* are reported using a circle. For all treatment groups, n=13.

As with data collected on caecal samples, the data collected on *C. jejuni* colonisation of the ileum was not normally distributed for all treatment groups and so the results are being discussed and presented as medians, including their respective interquartile range, given as lower (Q1) and Upper (Q3) quartile values. In accordance with the values collected for caecal content, the Internal control population had highest median $\log_{10}$ CFU/gram *C. jejuni* load values of 4.35, compared to 4.30 for Aviguard®, 3.85 for External control and 0.00 for CMT treatment groups. There was no significant variation between the load of *C. jejuni* found in the ileal content of Aviguard®, Internal and External control populations ($p > 0.05$). Since no birds that had been given CMT treatment showed ileal *C. jejuni* positivity, the CMT population had significantly lower *C. jejuni* colonisation of this part of the GIT compared to Aviguard® treated ($p = 0.0001$), Internal control ($p = 0.0005$) and External control ($p = 0.0016$) groups. Details of statistical parameters and group comparison significance values are provided in Table 15, with enumeration data for all individually identified experimental animals listed in Appendix 3.

Table 15. Statistical parameters and treatment group comparison significance values determined for Log_{10}CFU/g *C. jejuni* ileal colonisation

Treatment group	*C. jejuni* load (Log_{10} CFU/g)			Data normality (P value)	Statistical test used	Group comparison	*P*-Value
	Median	Quartiles					
		Q1	Q3				
CMT	0.00	0.00	0.00	N/A	Mann Whitney-U	Aviguard® Internal Control External Control	0.0001 0.0005 0.0016
Aviguard®	4.30	1.96	5.27	0.159	Mann Whitney-U	Internal Control External Control	0.8385 0.0547
Internal control	4.35	0.00	6.11	0.5171	Mann Whitney-U	External Control	0.2123
External control	3.85	0.00	4.07	0.0062	N/A	N/A	N/A

EXTRA-INTESTINAL SPREAD OF *C. JEJUNI*

A tissue samples from both the spleen and liver were collected from all birds at post-mortem 35 d.p.h (14 d.p.i). As with previous samples, all birds in the non-infected trial control groups were negative for *C. jejuni* colonisation and will not be discussed further in any detail. No birds having received CMT treatment were positive for *C. jejuni* in either spleen or liver tissue samples collected.

Aviguard® and External control groups showed highest frequency of splenic tissue *C. jejuni* infiltration with presence in 5/13 (38 %) of each population. Of the Internal control population, 3/13 (21 %) were positive for *C. jejuni* in the splenic tissue. Hepatic colonisation of Aviguard® treated birds was higher than seen for splenic tissue of this group, with 6/13 (46 %) positive for *C. jejuni* in liver tissue. Both Internal and External control groups showed lower liver *C. jejuni* colonisation than seen for splenic colonisation, with hepatic colonisation in 1/13 (8 %) and 4/13 (31 %) birds in respective groups. Figure 45 shows spleen and liver positivity per bird according to their unique identification number pre- and post- sample enrichment.

Treatment Group	Bird ID	Spleen tissue		Liver tissue	
		D	E	D	E
CMT Treated	1703				
	1704				
	1705				
	1706				
	1707				
	1710				
	1712				
	1713				
	1714				
	1715				
	1717				
	1718*				
	1719*				
Aviguard Treated	3651				
	3652*			■	
	3653	■	■	■	■
	3654*				
	3655		■	■	■
	3657				
	3659	■			
	3660				
	3661			■	■
	3662			■	■
	3663	■	■	■	■
	3664				
	3665		■		
Internal Control	226		■		
	227	■	■	■	■
	228				
	229				
	230				
	231*				
	232		■		
	233*				
	234				
	236				
	237				
	238				
	239				
External Control	802*		■	■	
	803				
	804				
	805				
	806				
	807	■			
	809				
	810		■		
	811	■	■	■	■
	812	■	■	■	■
	813				
	814*				■
	815				

Figure 45 Detection of *C. jejuni* within liver and splenic tissues of broiler chickens under experimental conditions based on the protocol listed for experiment 7. Red squares depict *C. jejuni* detection within a single sample, whereby 'D' indicates results are from direct plating of tissue homogenate and 'E' depicts results are from enriched samples. All birds showing '*' were directly infected as seeder birds. For all treatment groups, n=13.

To assess whether high caecal *C. jejuni* colonisation was a predictor for systemic bacterial spread, splenic and hepatic tissue *C. jejuni* positivity was related back to caecal *C. jejuni* load, with this being provided in Figure 46. As shown, there is no visibly clear relationship between caecal *C. jejuni* colonisation and the likelihood of systemic bacterial spread within this experimental trial.

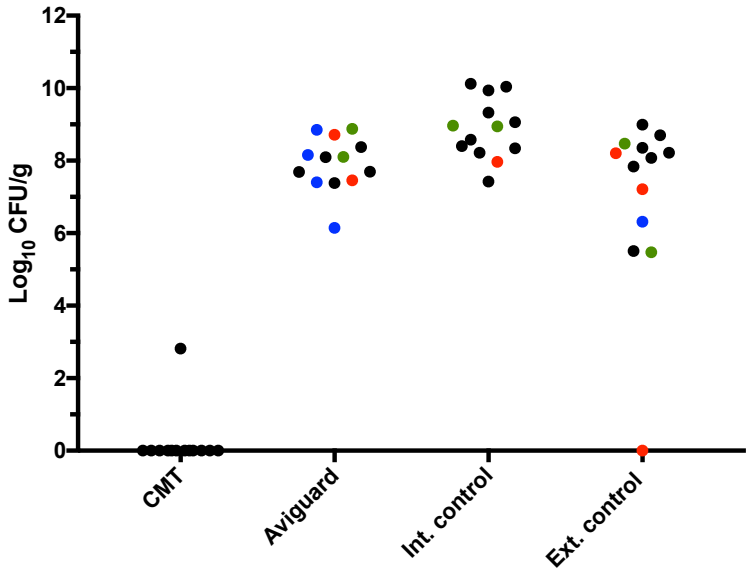


Figure 46. Extra-intestinal detection of *C. jejuni* mapped against *C. jejuni* caecal load for broiler chickens under experimental conditions based on experimental protocol 7. Each symbol represents results from an individual animal with caecal load given as Log_{10}CFU/g of caecal content. Green shapes indicate birds with *C. jejuni* detected in splenic tissue, blue shapes show detection in liver tissue and red shapes represent animals with *C. jejuni* detected in both splenic and liver tissues. For all treatment groups, n=13.

BODY WEIGHT

Immediately post-cull, whole carcasses of all birds were individually weighed to assess the impact of CMT or Aviguard® treatment on broiler weight gain. Data on bird weight was not normally distributed for all treatment groups and so results are discussed and presented as median values with their respective interquartile ranges (Figure 47). External control birds not hatched within our experimental unit showed highest overall group body weight, with a group median of 1020 g while birds hatched within our experimental unit and treated with CMT showed the lowest median body weight, being 805 g.

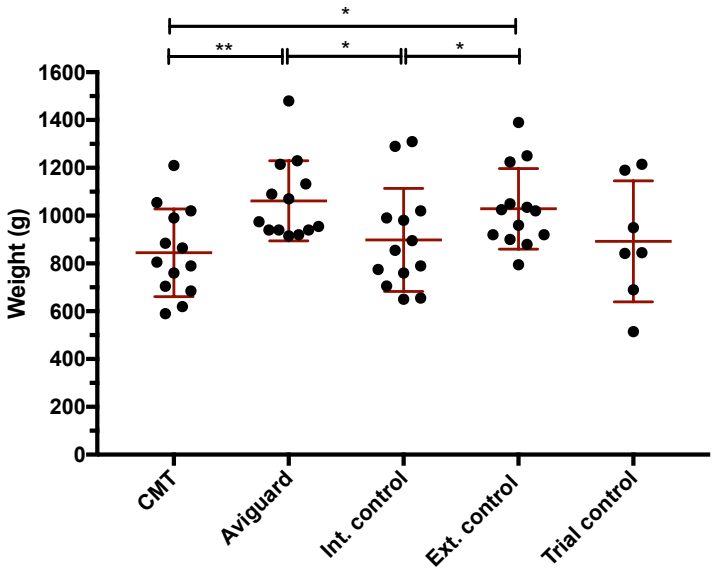


Figure 47. Whole body weight of broiler chickens based on the protocol listed for Experiment 7. Each symbol represents the body weight immediately post-cull for an individual animal. Statistical analysis is based on median values with associated IQR. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p < 0.05, **p < 0.01. For CMT, Aviguard, Int. control and Ext. control n=13, for Trial control n=7.

Aviguard® treated birds had significantly higher body weight than both CMT treated (p = 0.005) and Internal control (p = 0.044) birds, with all groups hatched within out experimental unit. CMT treated birds had significantly lower body weight compared to the External control group. External control birds had significantly higher overall body weight compared to that of the Internal control group, with hatchery environment being the only variation in treatment between these two groups. Statistical significance following group comparison is provided in Table 16.

Table 16. Statistical parameters and treatment group comparison significance values for bodyweight (g)

Treatment group	Body weight (g)			Data normality (P-Value)	Statistical test used	Group comparison	P-Value
	Median	Quartiles					
		Q1	Q3				
CMT	805	695	1005	0.6933	Mann Whitney-U	Aviguard®	0.005
						Internal Control	0.6405
						External Control	0.0124
						Trial Control	0.6992
Aviguard®	975	940	1174	0.0256	Mann Whitney-U	Internal Control	0.044
						External Control	0.544
						Trial Control	0.1181
Internal control	855	733	1005	0.3331	Mann Whitney-U	External Control	0.0455
						Trial Control	0.9385
External control	1020	910	1138	0.2757	Mann Whitney-U	Trial Control	0.1517
Trial control	845	690	1190	N/A	N/A	N/A	N/A

HUMORAL RESPONSE

Samples of 2 mL whole blood were collected from each bird via cardiac puncture at post-mortem 35 d.p.h (14 d.p.i). Serum samples were prepared and measured for specific IgM, IgA and IgY against *C. jejuni* using ELISA protocols detailed in Chapter 2. Data sets for all immunoglobulins tested were not normally distributed and so are discussed from this point onward in regard to median values and their respective IQR.

Across all treatment groups, median IgY levels were highest in the External control treatment group with an optical density at 405nm (OD_{405}) of 0.2185 (Figure 48a). The non-*C. jejuni* infected trial control group had the lowest median IgY, with a median OD_{405} of 0.1227, with this being significantly lower than that overserved in External control chickens (p = 0.0002). Median OD_{405} readings across the remaining three treatment groups (Internal control, CMT treated and Aviguard® treated) showed similar serum IgY levels, with median OD_{405} readings ranging from 0.155 – 0.1427. Serum IgY recorded for the External control treatment group were significantly higher than those of CMT treated (p = 0.0083), Aviguard® treated (p = 0.0083) and Internal control (p = 0.0242) experimental birds.

*C. jejuni s*pecific serum IgM was more strongly represented in each experimental group compared to IgY (Figure 48b). Serum IgM was significantly higher in all infected treatment groups compared to that found for non-infected trial control chickens (p < 0.0001). CMT treated birds had highest recoverable serum IgM titres, with median OD_{405} values of 0.2534, with this significantly higher than values observed for internal control birds (p < 0.05). Serum IgA titres were highest within samples of CMT treated birds, with median OD_{405} readings for this group being significantly higher than for all other treatment groups (p < 0.05) (Figure 48c). Details on statistical test parameters and group comparison significance are provided in Table 17.

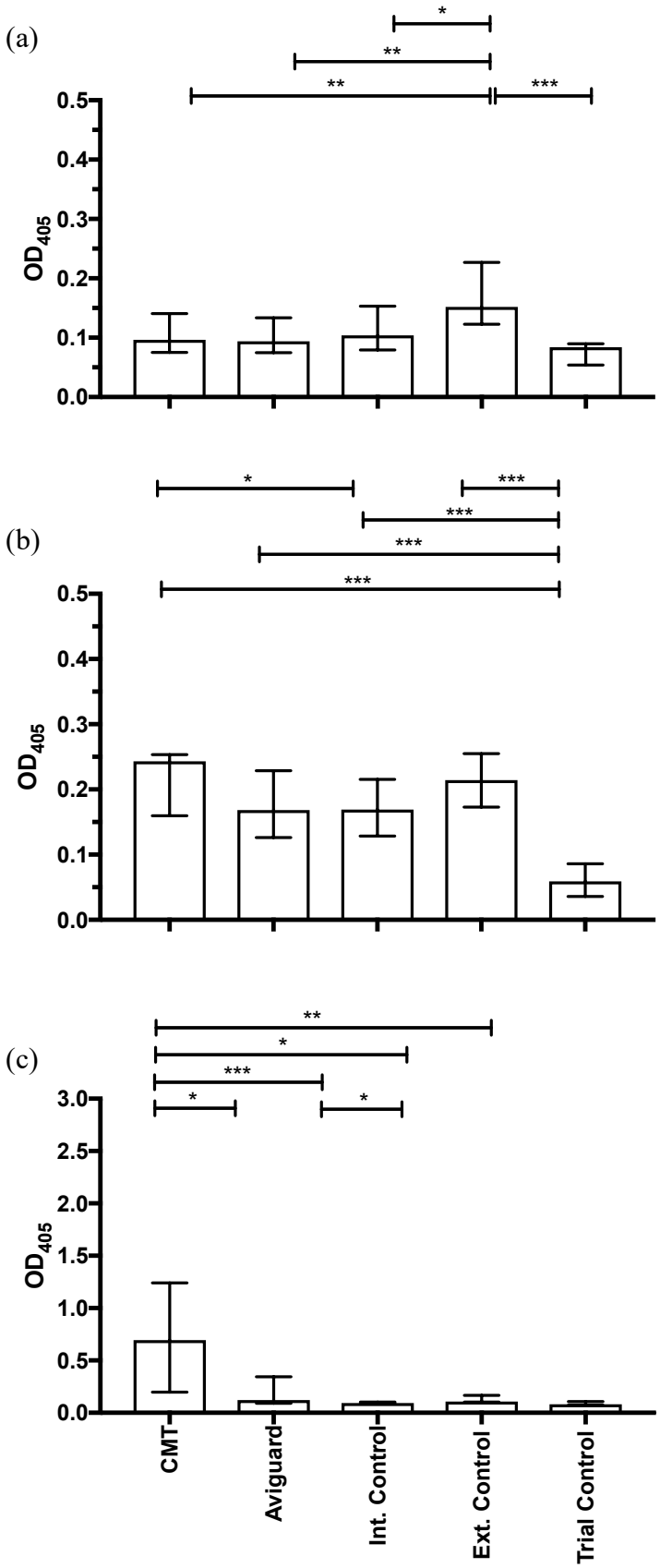


Figure 48. Serum (a) IgY (b) IgM and (c) IgA response to *C. jejuni* challenge and treatment group. Statistical analysis is based on median values with associated IQR. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p < 0.05, **p < 0.01, ***p < 0.001. For CMT, Aviguard, Int. control and Ext. control n=13, for Trial control n=7.

Table 17. Statistical parameters and treatment group comparison significance values determined for serum IgY, IgM and IgA (OD_{405})

Immunoglobulin	Treatment group	OD_{405}			Data normality (p -value)	Statistical test used	Group comparison	p-value
		Median	Quartiles					
			Q1	*Q3*				
IgY	CMT	0.097	0.075	0.1401	0.1208	Mann Whitney-U	Aviguard® Int. Control Ext. Control Trial Control	0.9323 0.5512 0.0083 0.2908
	Aviguard®	0.094	0.0749	0.134	<0.0001	Mann Whitney-U	Int. Control Ext. Control Trial Control	0.5137 0.0083 0.1505
	Int. Control	0.104	0.0794	0.153	0.4008	Mann Whitney-U	Ext. Control Trial Control	0.0242 0.0529
	Ext. Control	0.152	0.123	0.227	0.0029	Mann Whitney-U	Trial Control	0.0002
	Trial Control	0.0839	0.0539	0.0898	N/A	N/A	N/A	N/A
IgM	CMT	0.243	0.16	0.253	<0.0001	Mann Whitney-U	Aviguard® Int. Control Ext. Control Trial Control	0.0557 0.0233 0.6994 0.0005
	Aviguard®	0.168	0.126	0.229	<0.0001	Mann Whitney-U	Int. Control Ext. Control Trial Control	0.9487 0.0557 0.0009
	Int. Control	0.169	0.128	0.216	0.1793	Mann Whitney-U	Ext. Control Trial Control	0.0652 0.0005
	Ext. Control	0.214	0.173	0.255	0.077	Mann Whitney-U	Trial Control	0.0005
	Trial Control	0.059	0.036	0.0862	N/A	N/A	N/A	N/A
IgA	CMT	0.694	0.198	1.24	0.2115	Mann Whitney-U	Aviguard® Int. Control Ext. Control Trial Control	0.0242 0.0004 0.0145 0.0039
	Aviguard®	0.123	0.0915	0.343	0.0061	Mann Whitney-U	Int. Control Ext. Control Trial Control	0.1135 0.9323 0.0637
	Int. Control	0.0935	0.0893	0.104	0.0514	Mann Whitney-U	Ext. Control Trial Control	0.0173 0.3827
	Ext. Control	0.108	0.102	0.168	<0.0001	Mann Whitney-U	Trial Control	0.0637
	Trial Control	0.0825	0.0749	0.109	N/A	N/A	N/A	N/A

IN-VITRO STUDIES

Pre-exposure of avian intestinal epithelial cells to CMT or Aviguard® filtrate was assessed for its protective ability against invasion from two *C. jejuni* isolates (*C. jejuni* M1 and *C. jejuni* 13126) and one *S.* Typhimurium isolate (*Salmonella enterica subsp. enterica* serovar Typhimurium str. 4/74). Multiple repeat gentamicin invasion assays were completed for each bacterial strain, 3 for *C. jejuni* M1; 2 for *C. jejuni* 13126 and 2 for *S.* Typhimurium 4/74, with Figure 49 showing the average Log_{10} CFU/ml and percentage invasion for each group per challenge bacterial strain across all repeats. Appendix 3 details all enumeration data for each assay repeat per challenge strain.

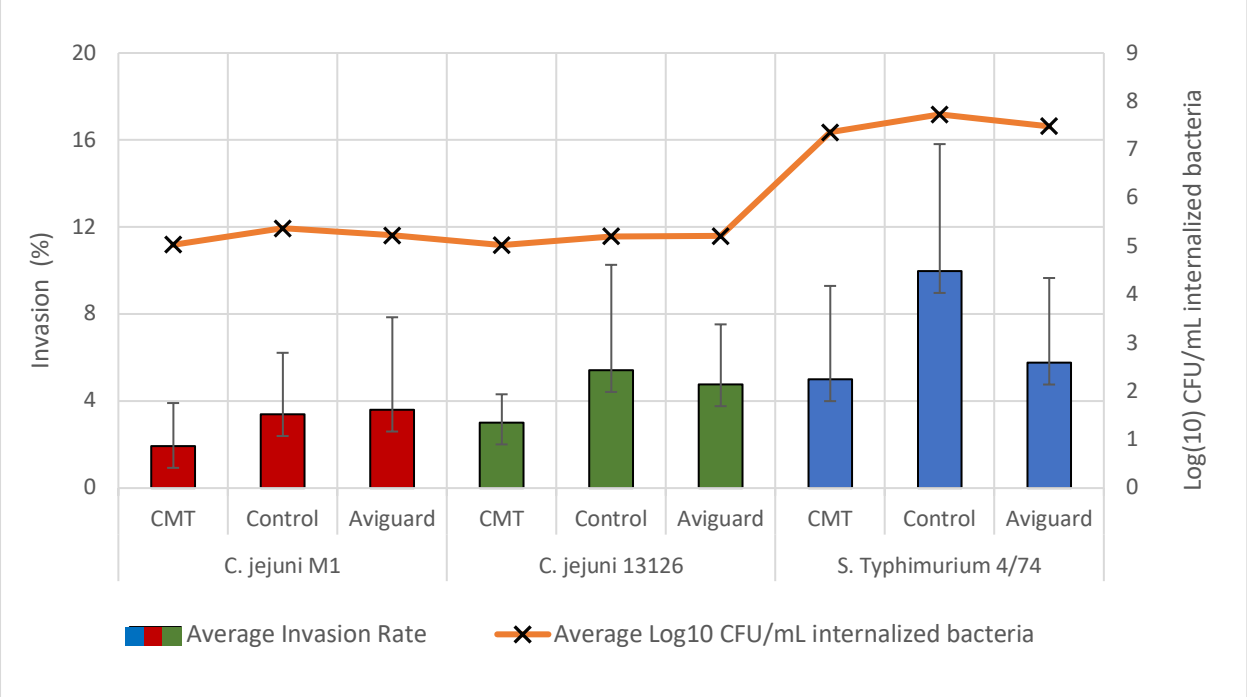


Figure 49. Invasion rate (%) and total internalised bacteria (Log_{10}CFU/ml) of all challenge strain following in-vitro GPA protocols. Results are shown as an average of all replicates for each GPA model. Error bars represent median values with their respective IQR.

GENTAMICIN PROTECTION ASSAY (GPA)

Bacteria internalized – C. jejuni M1

C. jejuni M1 invasion capacity was assessed *in-vitro* using three repeat gentamicin protection assays with an average infecting inoculum of 1.22 x 10^7 CFU/ml. Table 18 provides colony counts and levels of bacterial invasion of cultured 8E11 cells, with a complete assay

representing a 'Repeat' and each individual plate well seeded with 1 x 10^5 8E11 cells representing a 'Replicate'

We can first assess the recovered Log_{10} CFU/ml *C. jejuni* M1 for each of the three standalone assay repeats (Figure 50). Highest bacterial recovery was observed for all groups in Repeat 2 with Log_{10} CFU/ml ranging from 4.82 to 6.34 across treatment groups, reflecting the higher infecting inoculum (7.26 Log_{10}CFU/ml) applied in this assay compared to other repeats.

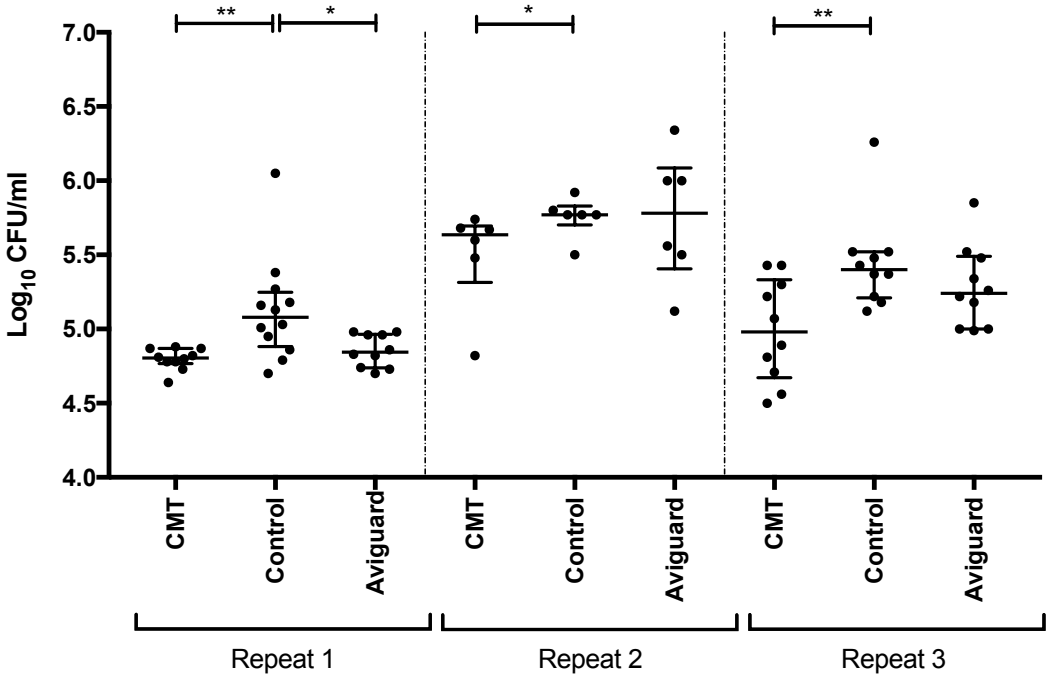


Figure 50. Recovered Log_{10}CFU/ml *C. jejuni* M1 following GPA protocols on 8E11 cells. Data is shown for each of 3 repeat assays with each point representing a single well replicate. Data is represented as median values with their associated IQR. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p < 0.05, **p < 0.01. Repeat 1; Control (n=12), CMT and Aviguard (n=10). Repeat 2; n=6 for all treatment groups; Repeat 3; n=10 for all treatment groups.

Cells pre-exposed to CMT filtrate prior to *C. jejuni* M1 infection showed significantly lower recoverable bacteria compared to MHB treated cells across all three assay repeats (Repeat 1 $p = 0.0041$, Repeat 2 $p = 0.0238$, Repeat 3 $p = 0.0093$). Cells pre-exposed to Aviguard® filtrate prior to *C. jejuni* M1 infection also showed significantly lower recoverable bacteria compared to MHB treated cells in assay repeat 1 ($p = 0.0195$), however this protection was non-reproducible in repeat 2 and 3 ($p = 0.4567$; $p = 0.2239$ respectively). Although recovered *C. jejuni* M1 was lower in cells pre-treated with CMT compared to those pre-treated with Aviguard® in all three assay repeats, this relationship was not found to be statistically

significant (Repeat 1 p = 0.27; Repeat 2 p = 0.8268; Repeat 3 p = 0.0928). Data-set normality and treatment group significance values are provided in Table 18.

Table 18. Statistical parameters and treatment group comparison significance values determined for Log_{10}CFU/ml *C. jejuni* M1 invasion of 8E11 cells using GPA protocols

Challenge strain	Assay repeat	Treatment group	Recovered *C. jejuni* (log_{10}CFU/ml)			Data normality (p-value)	Statistical test used	Group comparisons	P-value
			Median	**Quartiles**					
				Q1	**Q3**				
***C. jejuni* M1**	1	Control (n=12)	5.08	4.88	5.25	0.0031	Mann Whitney-U	Control v CMT	0.0041
		CMT (n= 10)	4.81	4.77	4.87	0.1627		Control v Aviguard®	0.0195
		Aviguard® (n= 10)	4.85	4.74	4.97	0.2272		CMT v Aviguard®	0.27
	2	Control (n= 6)	5.77	5.70	5.83	N/A	Mann Whitney-U	Control v CMT	0.0238
		CMT (n=6)	5.64	5.32	5.7	N/A		Control v Aviguard®	0.4567
		Aviguard® (n=6)	5.78	5.41	6.09	N/A		CMT v Aviguard®	0.8268
	3	Control (n=10)	5.40	5.21	5.52	0.0007	Mann Whitney-U	Control v CMT	0.0093
		CMT (n=10)	4.98	4.67	5.33	0.3362		Control v Aviguard®	0.2239
		Aviguard® (n=10)	5.24	5.00	5.49	0.3695		CMT v Aviguard®	0.0928

Bacteria internalized – C. jejuni 13126

C. jejuni 13126 invasion capacity was assessed in vitro using two repeat gentamicin protection assays with an average infecting inoculum of 4.17 x 10^6 CFU/ml (Figure 51). Assessing Log_{10}CFU/ml *C. jejuni* 13126 for each assay repeat, highest recovery for all treatment groups was in assay repeat 1, with an overall Log_{10} CFU/ml ranging from 5.07 to 6.03. As with *C. jejuni* M1, this could represent the higher infecting *C. jejuni* 13126 inoculum used in assay repeat 1 compared to assay repeat 2.

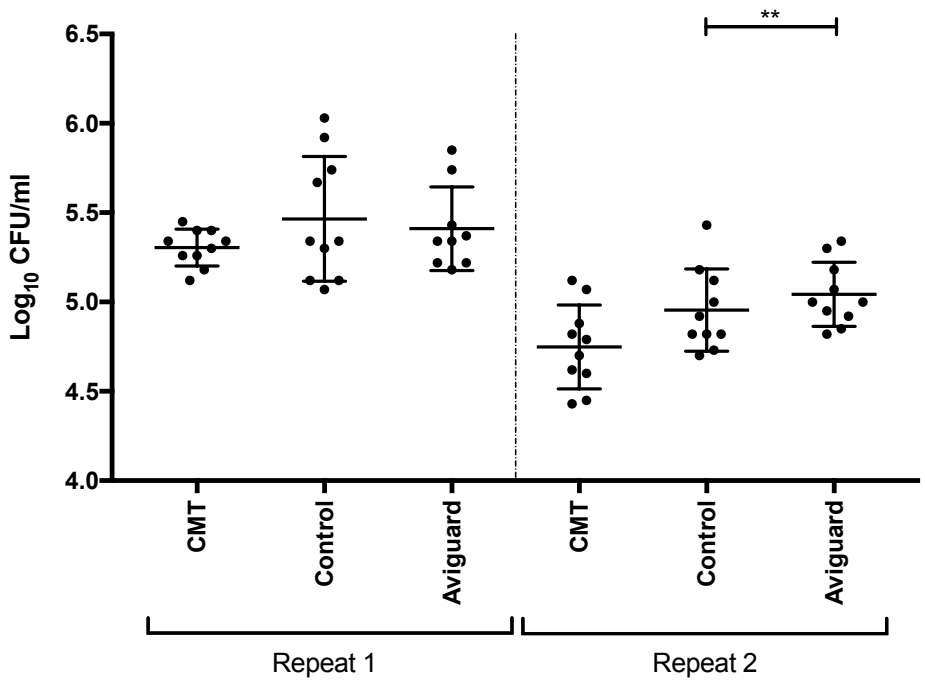


Figure 51. Recovered Log_{10}CFU/mL C. jejuni 13126 following GPA protocols on 8E11 cells. Data is shown for each of 2 repeat assays with each point representing a single well replicate. Data is represented as mean values with their associated SD. Statistical significance was determined using Unpaired t-test analysis with levels of significance given as **p < 0.01. Repeat 1; CMT and Control (n=10), Aviguard (n=9), Repeat 2; n=10 for all treatment groups.

Cells pre-treated with CMT filtrate had the lowest level of recoverable *C. jejuni* 13126 in both assay repeat 1 (5.305 Log_{10}CFU/ml) and assay repeat 2 (4.75 Log_{10}CFU/ml). Treatment group showed no significant effect on *C. jejuni* 13126 internalization in assay repeat 1 ($p > 0.05$), while in repeat 2, prophylactic CMT filtrate treated reduced challenge strain internalization compared to prophylactic Aviguard® treatment ($p = 0.0054$). Interestingly in assay repeat 2, cells pre-treated with Aviguard® filtrate showed higher *C. jejuni* 13126 recovery following lysis

compared to non-treated cells, however this was not statistically significant (p = 0.3472). Data set normality and treatment group significance values are provided in Table 19.

Table 19. Statistical parameters and treatment group comparison significance values determined for Log_{10}CFU/ml C. jejuni 13126 invasion of 8E11 cells using GPA protocols

Challenge strain	Assay repeat	Treatment group	Average log_{10} CFU/ml ± SD	Data normality (p-value)	Statistical test used	Group comparisons	P-value
C. Jejuni 13126	1	Control (n=10)	5.47 ± 0.35	0.4024	Unpaired t-test	Control v CMT	0.1814
						Control v Aviguard®	0.6954
		CMT (n=10)	5.31 ± 0.10	0.7805		CMT v Aviguard®	0.2149
		Aviguard® (n=9)	5.41 ± 0.23	0.2365			
	2	Control (n=10)	4.95 ± 0.23	0.2788	Unpaired t-test	Control v CMT	0.0632
						Control v Aviguard®	0.3472
		CMT (n=10)	4.75 ± 0.24	0.7963		CMT v Aviguard®	0.0054
		Aviguard® (n=10)	5.04 ± 0.18	0.5849			

Bacterial internalized - S. Typhimurium *4/74*

S. Typhimurium 4/74 invasion capacity was assessed in vitro using two repeat gentamicin protection assays with an average infecting inoculum of 6.58 x 10^8 CFU/ml. Between assay repeat variation was less defined for *S.* Typhimurium assays compared to those of *C. jejuni*, with treatment groups from both assay repeats showing similarly recoverable levels of *S.* Typhimurium. Overall Log_{10} CFU/ml ranged from 6.52 to 7.91 in assay repeat 1 and 7.07 to 8.18 in assay repeat 2. The high bacterial recovery rate obtained in both *S.* Typhimurium assays was likely due to the higher bacterial CFU/ml in the inoculating infection material, compared to that used for *C. jejuni* infection protocols.

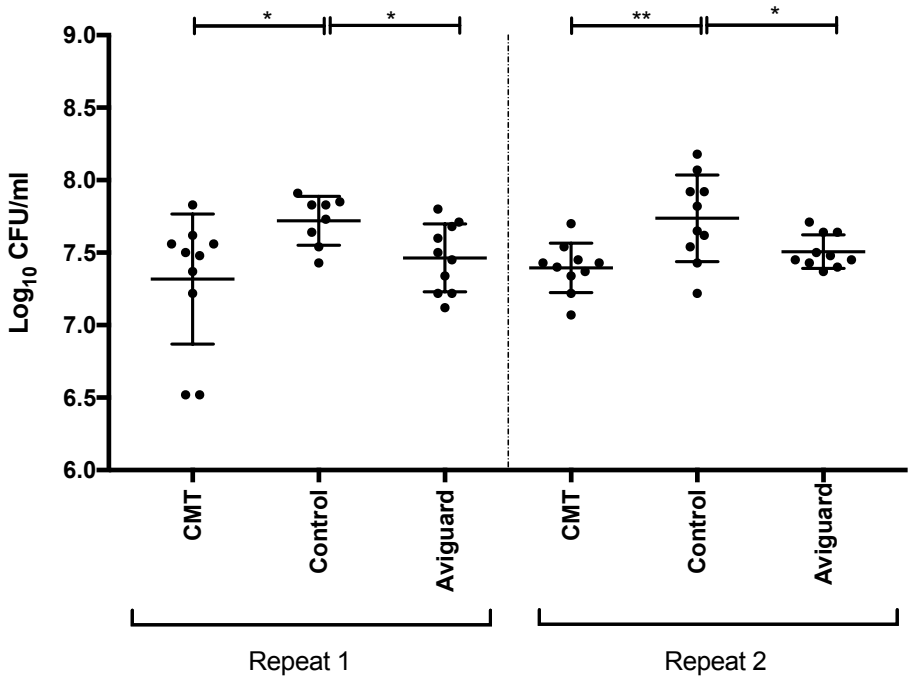


Figure 52. Recovered Log_{10}CFU/mL S. Typhimurium 4/74 following GPA protocols on 8E11 cells. Data is shown for each of 2 repeat assays with each point representing a single well replicate. Data is represented as mean values with their associated SD. Statistical significance was determined using Unpaired t-test analysis with levels of significance given as *p < 0.05, **p < 0.01. Repeat 1; CMT and Aviguard (n=10), control (n=8), Repeat 2; n=10 for all treatment groups.

Cells pre-treated with CMT filtrate had significantly lower recoverable *S.* Typhimurium 4/74 levels compared to LB-treated control cells in both assay repeat 1 (p = 0.0296) and assay repeat 2 (p = 0.0056). Similarly, this relationship was also evident for cells pre-treated with Aviguard® filtrate, showing significantly reduced *S.* Typhimurium levels in both assays compared to untreated control cells (Repeat 1 p = 0.0196; Repeat 2 p = 0.0356). Although cells pre-treated with CMT showed lower recoverable *S.* Typhimurium compared to pre-treated Aviguard® cells, this was not statistically significant for either assay repeat 1 (p = 0.374) or repeat 2 (p = 0.1017) (Figure 52). Data set normality and treatment group significance values are provided in Table 20.

Table 20. Statistical parameters and treatment group comparison significance values determined for Log_{10}CFU/ml *S.*Typhimurium 4/74 invasion of 8E11 cells using GPA protocols

Challenge strain	Assay repeat	Treatment group	Average log_{10} CFU/ml ± SD	Data normality (p value)	Statistical test used	Group comparison	P value
S. Typhimurium *4/74*	1	Control (n=8)	7.72 ± 0.17	0.5649	Unpaired t-test	Control v CMT	0.0296
						Control v Aviguard®	0.0196
		CMT (n=10)	7.32 ± 0.45	0.1651		CMT v Aviguard®	0.374
		Aviguard® (n=10)	7.46 ± 0.23	0.449			
	2	Control (n=10)	7.74 ± 0.30	0.8919	Unpaired t-test	Control v CMT	0.0056
						Control v Aviguard®	0.0356
		CMT (n=10)	7.40 ± 0.17	0.5555		CMT v Aviguard®	0.1017
		Aviguard® (n=10)	7..51 ± 0.12	0.4523			

Percentage invasion

Percentage invasion was calculated for each challenge strain to show the number of invading bacteria (CFU/ml) as a percentage of the number of bacterium (CFU/ml) in the infecting inoculum, with percentage invasion values per replicate provided in Appendix 3. Standardizing the data in this way allowed for the merging of percentage invasion data from each assay repeat to form one individual data set. Prior to pairwise comparison analysis, datasets were assessed for normality using a D'Agostino & Pearson normality test. Since non-normal distribution was not confirmed for all datasets, description will be given according to median values and respective IQR.

The range of *C. jejuni* M1 percentage invasion across well replicates was appreciably higher in Control cells (47.48%) compared to both Aviguard® (17.91%) and CMT (6.66 %) treated cells. Average cell invasion of *C. jejuni* M1 was significantly lower in cells pre-treated with CMT (1.93%) compared to non-treated control cells (5.01%) (p = 0.00177). Although average cell invasion percentage was lower in cells pre-treated with CMT compared to those pre-treated with Aviguard® filtrate (3.60%), this was not of statistical significance (p = 0.0952). There was no significant reduction in *C. jejuni* M1 invasion of Aviguard® filtrate treated cells compared to non-treated control cells (p = 0.1947)(Figure 53). Median invasion percentage and significance values per treatment group are provided in Table 21.

Although less drastic, as with *C. jejuni* M1 the range of *C. jejuni* 13126 percentage invasion across well replicates was higher in Control cells (17.94%) compared to both Aviguard® filtrate (10.38%) and CMT filtrate (4.21%) treated cells. Neither cells treated with CMT filtrate nor Aviguard® filtrate showed significant reduction in invasion percentage of *C. jejuni* 13126 (p = 0.1184; p = 0.9605 respectively). CMT filtrate pre-treatment offered significantly better protection from *C. jejuni* 13126 invasion compared to pre-treatment of cells with Aviguard® filtrate (p = 0.0309)(Figure 53). Median invasion percentage and significance values per treatment group are provided in Table 21.

Unlike both *Campylobacter* challenge strains, replicates with high levels of *S.* Typhimurium invasion were seen across all treatment groups with invasion percentage ranges of 19.51 % for Control cells, 16.95 % CMT treated cells and 14.02 % for Aviguard® treated cells. Pre-treatment of cells with both Aviguard® and CMT material significantly reduced the ability of *S.* Typhimurium to invade 8E11 cells compared to LB treated control cells (p = 0.0177; p = 0.0038 respectively). Median invasion percentage and significance values per treatment group are provided in Table 21.

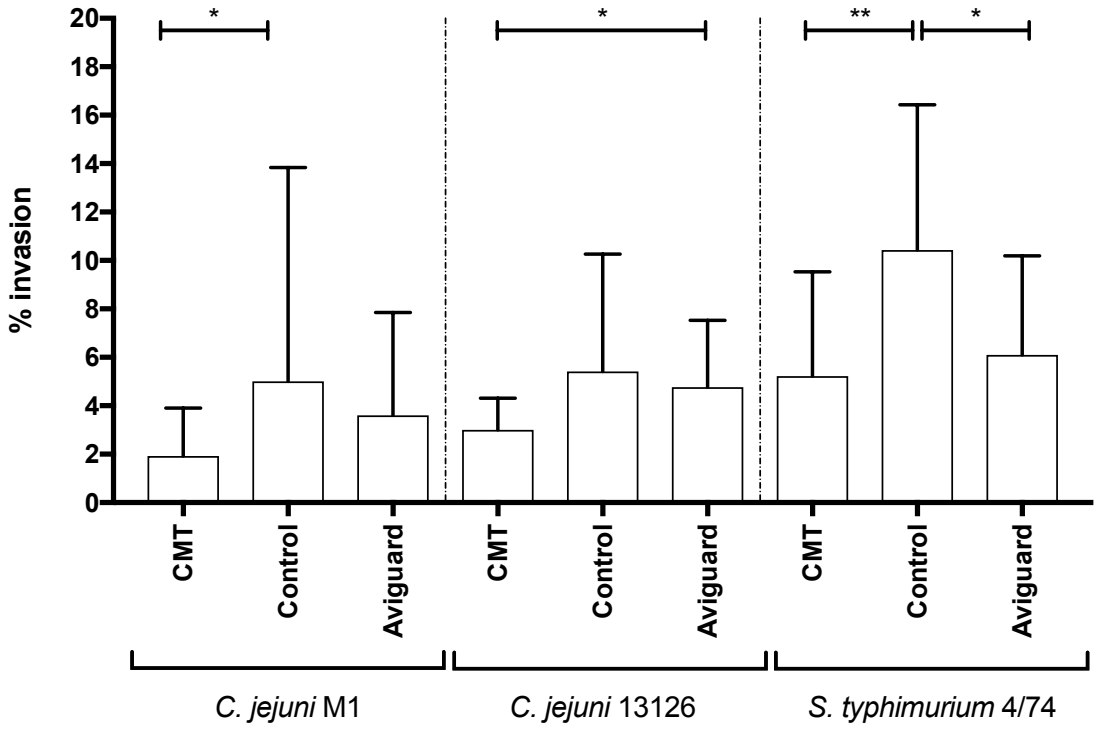


Figure 53. Invasion percentage of challenge bacterial strains on 8E11 cells according to treatment group. Data is represented as median values with their associated IQR. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p < 0.05, **p < 0.01.

Table 21. Statistical parameters and treatment group comparison significance values determined for challenge strain invasion percentage of 8E11 cells using GPA protocols

Challenge strain	Treatment group	Invasion (%)			Data normality (p value)	Statistical test used	Group comparison	P value
		Median	Q1	Q3				
***C. jejuni* M1**	Control (n= 27)	3.18	0.940	6.09	<0.0001	Mann Whitney-U	Control v CMT	0.0177
							Control v Aviguard®	0.4714
	CMT (n= 26)	1.16	0.448	2.73	0.0036		CMT v Aviguard®	0.148
	Aviguard® (n= 26)	2.29	0.605	5.45	<0.0001			
***C. jejuni* 13126**	Control (n=20)	3.59	2.35	7.68	0.0004	Mann Whitney-U	Control v CMT	0.1798
							Control v Aviguard®	0.4228
	CMT (n=20)	3.03	1.87	4.08	0.2417		CMT v Aviguard®	0.0309
	Aviguard® (n=19)	3.94	3.03	5.29	0.0007			
***S.* Typhimurium 4/74**	Control (n=18)	9.03	4.73	16.51	0.3115	Mann Whitney-U	Control v CMT	0.0038
							Control v Aviguard®	0.2131
	CMT (n=20)	3.40	2.37	8.15	0.0042		CMT v Aviguard®	0.0177
	Aviguard® (n=20)	4.50	3.08	8.04	0.0196			

Invasion ability of challenge bacterial strains

Identification of strain variation in in-vitro avian intestinal cell invasion ability was demonstrated through comparison of percentage bacterial invasion of untreated control cells between each inoculated bacterial strain. We were then able to infer how the protective ability of CMT and Aviguard® filtrate may be impacted by infecting bacterial invasive capability *in-vitro*. Figure 54 shows percentage cell invasion of un-treated control replicates for each bacterial strain, with results from statistical comparisons being provided in Table 22.

Table 22. Statistical parameters and challenge strain comparison significance values determined for invasion percentage of 8E11 cells using GPA protocols

Challenge strain	Invasion (%)			Data normality (p-value)	Statistical test used	Group comparison	P-value
	Median	Q1	Q3				
C. jejuni M1	3.18	0.955	6.09	<0.0001	Mann Whitney-U	*C. jejuni* 13126	0.14
						S. Typhimurium 4/74	<0.0001
C. jejuni 13126	3.59	2.35	7.68	0.0004	Mann Whitney-U	*C. jejuni* 13126 v *S.* Typhimurium 4/74	0.0047
S. Typhimurium 4/74	9.03	4.73	16.51	0.3115			

Looking at percentage cell invasion to take into account for between assay variation in infection inoculum bacterial load*, S.* Typhimurium was found to be the most invasive bacterial strain tested on our 8E11 cultured cells. Invasion capability of *S.* Typhimurium was significantly higher than both *C. jejuni* M1 ($p < 0.0001$) and *C. jejuni* 13126 ($p = 0.0047$). There was no significant variation in the invasion ability between the tested *C. jejuni* strains ($p = 0.14$) on our cultured avian epithelial cell line.

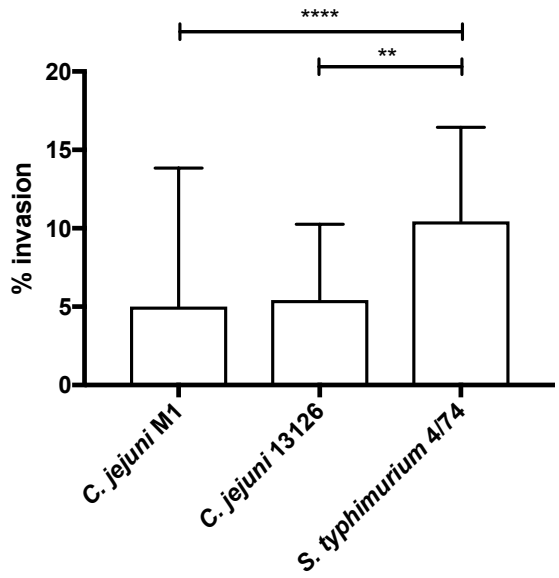


Figure 54. Invasion percentage of challenge bacterial strains on 8E11 cells. Data is represented as median values with their associated IQR. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as **p < 0.01, ****p < 0.0001. *C. jejuni* M1 n=27; *C. jejuni* 13126 n=20, *S.* typhimurium n=18.

NITRIC OXIDE PRODUCTION ASSAY

C. jejuni M1

Griess reagent was used to assess nitrite production from cells following 4 - or 24-hour infection with *C. jejuni* M1 or S. Typhimurium 4/74. Minimal concentrations of Nitrite were recovered from cells following 4-hour incubation with *C jejuni*, with median values ranging from 2.16 – 2.51 (IQR 1.54) across all infected cell groups. Incubation with *C. jejuni* over a 24 hour period increased the maximal recoverable nitrite concentrations within all infected cell groups, with median recovery of 12.74 – 15.28 (IQR 5.93) (Figure 55a). Nitrite release from non-infected cells at 24 hours incubation showed no significant increase compared to that of 4-hour incubation, indicating no underlying trend of increasing Nitrite release from cells over time.

Assessing Nitrite release from cells in respect to specific treatment groups showed, at 4 hours post infection, Aviguard® treated and infected control cells had significantly higher release of Nitrite compared to cells not infected with *C. jejuni* (Figure 55b). However, median Nitrite release from both groups remained comparably low being 2.44 (IQR 1.11) and 2.51 (IQR 0.31) respectively. Cells treated with CMT filtrate showed no significant increase in Nitrite release compared to non-infected cells ($p = 0.1797$). A longer, 24 hour incubation period significantly increased recoverable nitrite in all *C. jejuni* infected groups compared to non-infected cells ($p < 0.05$) (Figure 55c). Cells prophylactically treated with Aviguard® filtrate had highest median Nitrite release (15.28; IQR 3.66) compared to those treated with CMT filtrate (12.77; IQR 3.75) and infected control cells (12.74; IQR 2.09) ($p = 0.021$; $p = 0.0019$) Figure 55c. Data set normality and comparison significance values are provided in Table 23.

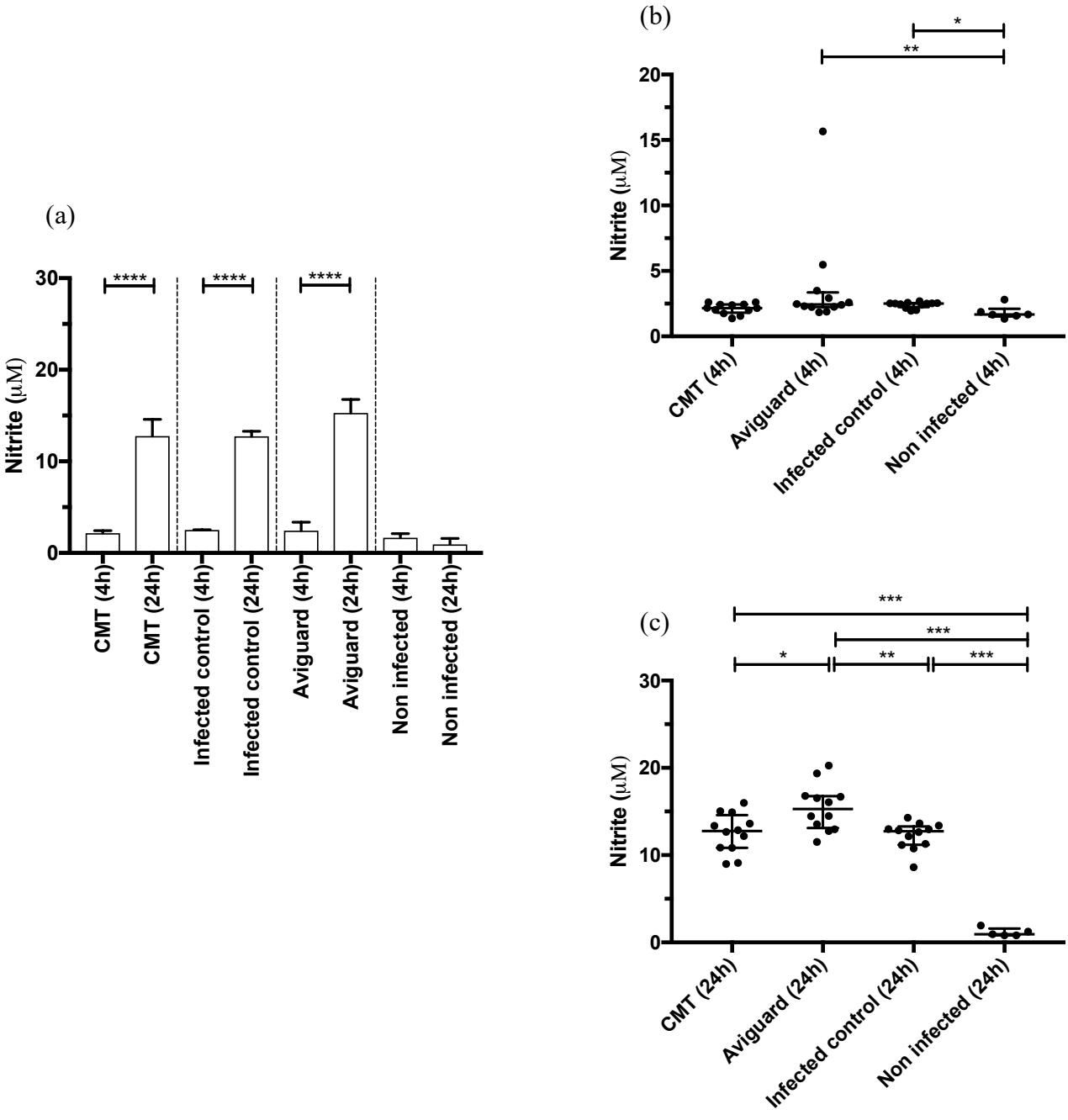


Figure 55. Effects of treatment on the concentration of nitrites in 8E11 cell supernatant after challenge with *C. jejuni* M1. Data is represented as median values with their associated IQR. Pairwise comparisons have been made for (a) challenge duration (b) effect of treatment group after 4-hour challenge (c) effect of treatment group after 24-hour challenge. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. For CMT, Aviguard and Inf. Control (n=12); Non infected control n=6.

S. Typhimurium *4/74*

Nitrite recovery from cell supernatant followed similar trends for all treatment groups when using infection strain *S.* Typhimurium as with *C. jejuni*. Low median nitrite concentrations were recovered from all infected groups at 4 hours post infection, ranging from 3.17 - 4.072 (IQR 2.37), with significantly higher nitrite at 24 hours post infection, ranging from 34.23 – 44.54 (IQR 18.67) ($p < 0.05$) (Figure 56a). Nitrite release from non-infected cells at 24 hours incubation showed no significant increase compared to that of 4 hour incubation ($p = 0.0823$), indicating no underlying trend of increasing Nitrite release from cells over time.

Assessing effects of specific treatment group against nitrite release at 4 hours post infected, all infected treatment groups released significantly more nitrite than cells that were not infected with *S.* Typhimurium (Figure 56b). Infected control cells released significantly higher nitrite compared to CMT treated cells ($p = 0.0205$). Incubation with *S.* Typhimurium over 24 hours significantly increased recoverable nitrite in infected cell groups compared to non-infected cells ($p < 0.05$) (Figure 56c). Cells pre-treated with Aviguard® had highest median nitrite release (44.54; IQR 13.94), with this being significantly higher than that of CMT treated cells ($p = 0.0242$). Data set normality and comparison significance values are provided in Table 23.

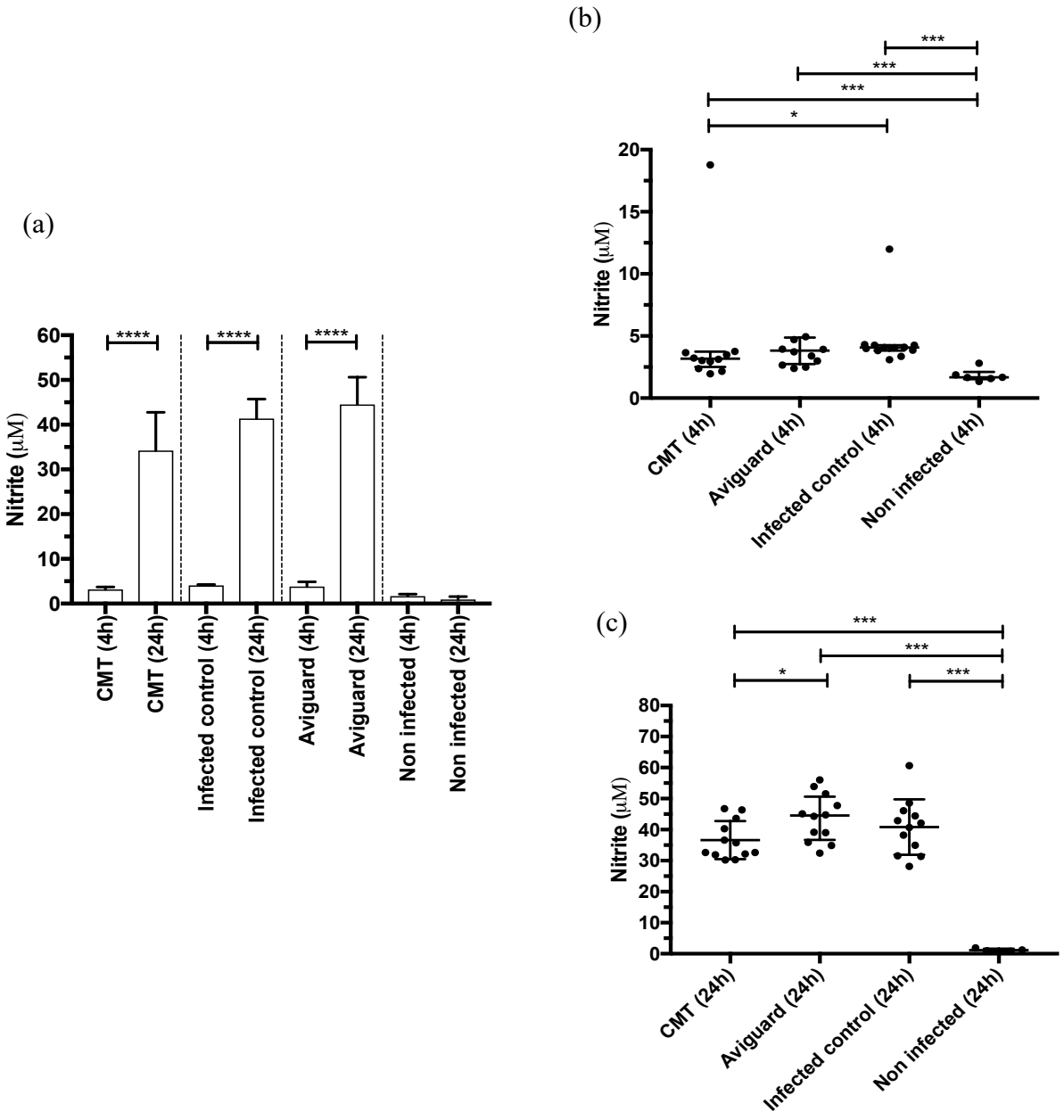


Figure 56. Effects of treatment on the concentration of nitrites in 8E11 cell supernatant after challenge with S. Typhimurium 4/74. Data is represented as median values with their associated IQR. Pairwise comparisons have been made for (a) challenge duration (b) effect of treatment group after 4-hour challenge (c) effect of treatment group after 24-hour challenge. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p<0.05, ***p<0.001, ****p<0.0001. For CMT, Aviguard and Inf. Control (n=12); Non infected control n=6.

Table 23. Statistical parameters and treatment group comparison significance values determined for per treatment group Nitrite (µM) release from 8E11 cells following 4 – or 24 hours challenge strain incubation

Challenge incubation	Challenge strain	Treatment group	Nitrite Release (µM)			Group comparison	P-value
			Median	Q1	Q3		
4 hour	*C. jejuni M1*	Control	2.51	2.24	2.54	CMT	0.0661
						Aviguard®	0.8096
						Non-infected	0.0232
		CMT	2.16	1.81	2.44	Aviguard®	0.078
						Non-infected	0.1797
		Aviguard®	2.44	2.25	3.35	Non-infected	0.0097
		Non-infected	1.67	1.52	2.1		
	S. Typhimurium 4/74	Control	4.07	3.83	4.26	CMT	0.0205
						Aviguard®	0.4428
						Non-infected	0.0001
		CMT	3.17	2.51	3.74	Aviguard®	0.2657
						Non-infected	0.0008
		Aviguard®	3.83	2.74	4.88	Non-infected	0.0008
		Non-infected	1.67	1.52	2.1		
24 hour	*C. jejuni M1*	Control	12.74	11.2	13.29	CMT	0.7125
						Aviguard®	0.0019
						Non-infected	0.0003
		CMT	12.77	10.84	14.59	Aviguard®	0.0121
						Non-infected	0.0003
		Aviguard®	15.28	13.11	16.77	Non-infected	0.0003
		Non-infected	0.94	0.809	1.58		
	S. Typhimurium 4/74	Control	41.4	32.39	45.71	CMT	0.3474
						Aviguard®	0.3474
						Non-infected	0.0003
		CMT	34.23	31.96	42.79	Aviguard®	0.0242
						Non-infected	0.0003
		Aviguard®	44.54	36.69	50.63	Non-infected	0.0003
		Non-infected	0.94	0.81	1.58		

NO production by challenge strain

By comparing release of nitrite from infected control cells of both *C. jejuni M1* and S. Typhimurium 4/74 infection models, we can assess the differential ability of each bacterial strain to induce nitrite response. After both 4- and 24 hours of incubation with each challenge strain. S. Typhimurium showed significantly higher nitrite response compared to *C. jejuni*, with significance values of $p < 0.0001$ (Figure 57).

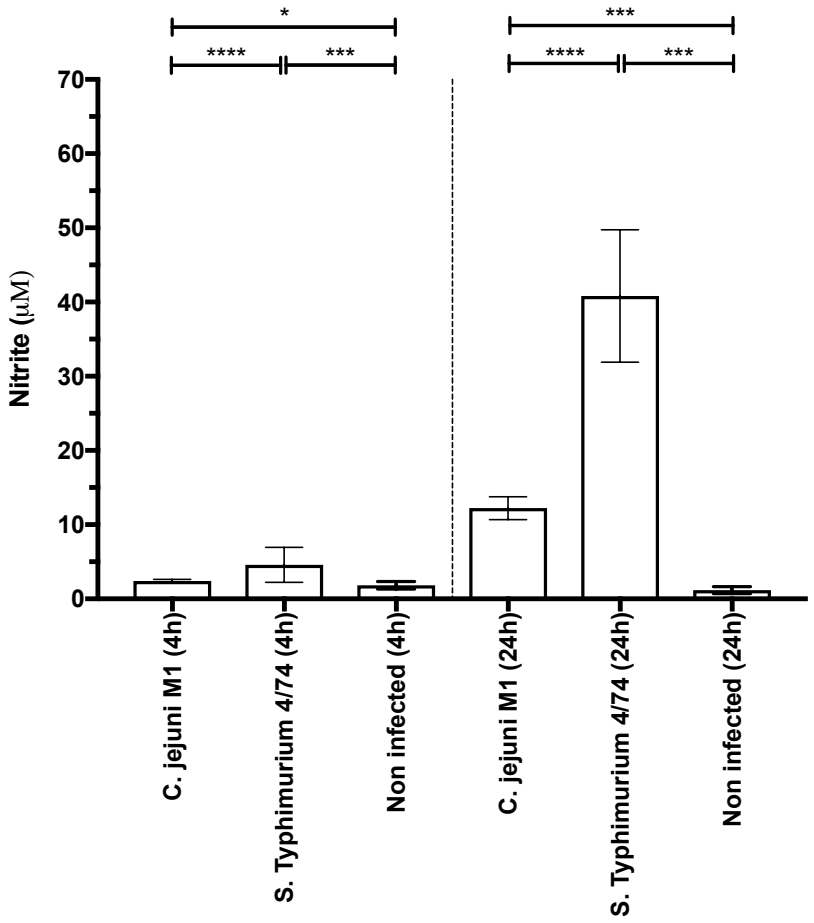


Figure 57. Effects of challenge strain on the concentration of nitrites in 8E11 cell supernatant. Data is represented as median values with their associated IQR. Pairwise comparisons have been made for (a) challenge duration (b) effect of treatment group after 4-hour challenge (c) effect of treatment group after 24-hour challenge. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p<0.05, ***p<0.001, ****p<0.0001. For each challenge strain CMT, Aviguard and Inf. Control (n=12); Non infected control n=6.

GENE EXPRESSION BY $2^{-\Delta\Delta CT}$ RT-qPCR: 4 HOUR CHALLENGE

Pre-treatment of cultured avian 8E11 epithelial cells with CMT or Aviguard® treatment was investigated for its ability to influence inducible immune gene expression. Expression of IL-1ß, IL-6, IL-10, TGFß$_4$, CXCLi1, CXCLi2, Mucin2 and AvBD9 mRNA was measured following RNA extraction from treated 8E11 cell lines (Figure 40) at 4 hours post infection with either *C. jejuni* or *S.* Typhimurium. For both invasion models, I was unable to attain reliable qRT-PCR reaction data using IL-1ß and IL-10 primers and as such, the data presented within this chapter will excluded both IL-1ß and IL-10 cytokine expression values. All data presented was first assessed for normality of distribution using D'Agostino & Pearson analysis. Since all data did not adhere to a normal distribution ($p < 0.05$), description will be based upon median values and their respective IQR according to Mann Whitney-U comparison-based analysis with a significance threshold of $p < 0.05$. Each cell culture well was designated as a single replicate, with 10 replicates per treatment group, per immune gene. Non-infected control groups were represented by six replicates.

C. jejuni M1

IL-6 expression significantly increased within all infected groups compared to that of the non-infected control cells, with CMT treated cells showing highest up-regulation of 5.08 – fold (Figure 59 & Figure 60). The chemokine ligand CXCLi1 showed lesser tendency toward upregulation following *C. jejuni* infection compared to that of IL-6 with fold change ranging from 0.17 - 1.81 across all treatment groups compared to non-infected cells. Treatment group had no effect on CXCLi1 expression ($p > 0.05$). A secondary chemokine ligand, CXCLi2 showed tendency toward downregulation within both Infected control and Aviguard® treated cell groups compared to non-infected cells, while CMT treated cells showed upregulation of 2.19 - fold. Expression of CXCLi2 was significantly higher in CMT treated cells compared to Aviguard® treated ($p = 0.0012$) and infected control cells ($p = 0.0047$). *C. jejuni* infected control cells showed minimal change in TGFß$_4$ expression compared to that of non-infected cells, with an overall 1.04 - fold change observed. Expression of TGFß$_4$ was significantly upregulated in CMT treated cell populations compared to all other treatment groups ($p < 0.01$), while Aviguard® treated cells showed significant upregulation compared to only those cells not infected with *C. jejuni* M1 ($p < 0.05$).

Expression of Mucin 2 (MUC2) was largely consistent across cells of all treatment groups 4 hours post *C. jejuni* infection, with only CMT treated cells showing any marked variability. Expression of MUC2 in CMT treated cells was significantly upregulated compared to *C. jejuni* infected control cells (p = 0.0070). Conversely, avian beta-defensin 9 (AvBD9) expression showed apparent modulation according to specific treatment group. All groups infected with *C. jejuni* had increased transcription of AvBD9 compared to non-infected cell replicates. Greatest upregulation of AvBD9 was recorded within the CMT group compared to all other cell treatment groups ($p < 0.05$) with a 4.81-fold increase compared to non-infected cells. Statistical analysis parameters and p - values for all treatment group comparisons are provided in Table 24.

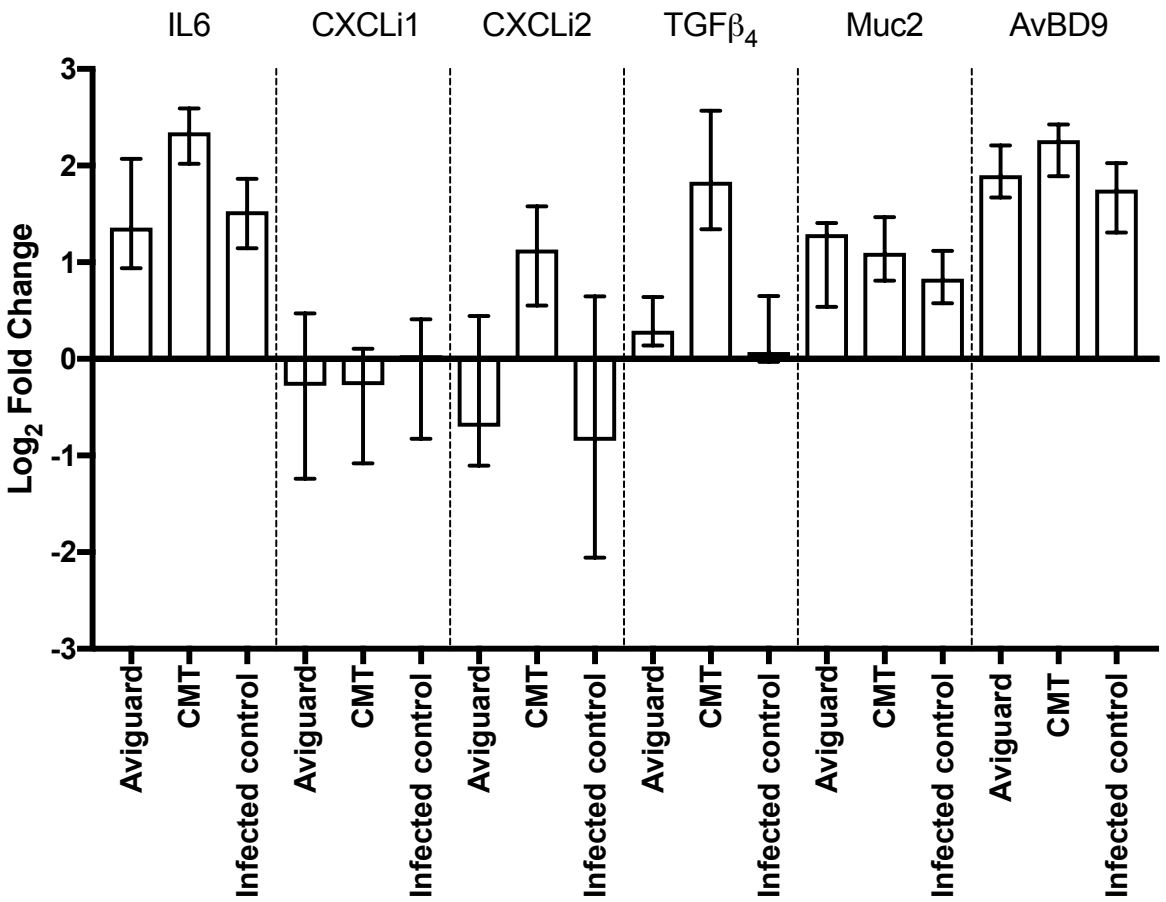


Figure 58. Log_2 relative expression of IL-6, CXCLi1, CXCLi2, TGFß4, MUC2 and AvBD9 following RNA extraction from 8E11 cells challenged with *C. jejuni* M1 over 4-hours compared to non-challenged cells. Data is represented as median values with respective IQR. For all treatment groups, n=10; for non-infected control groups n=6.

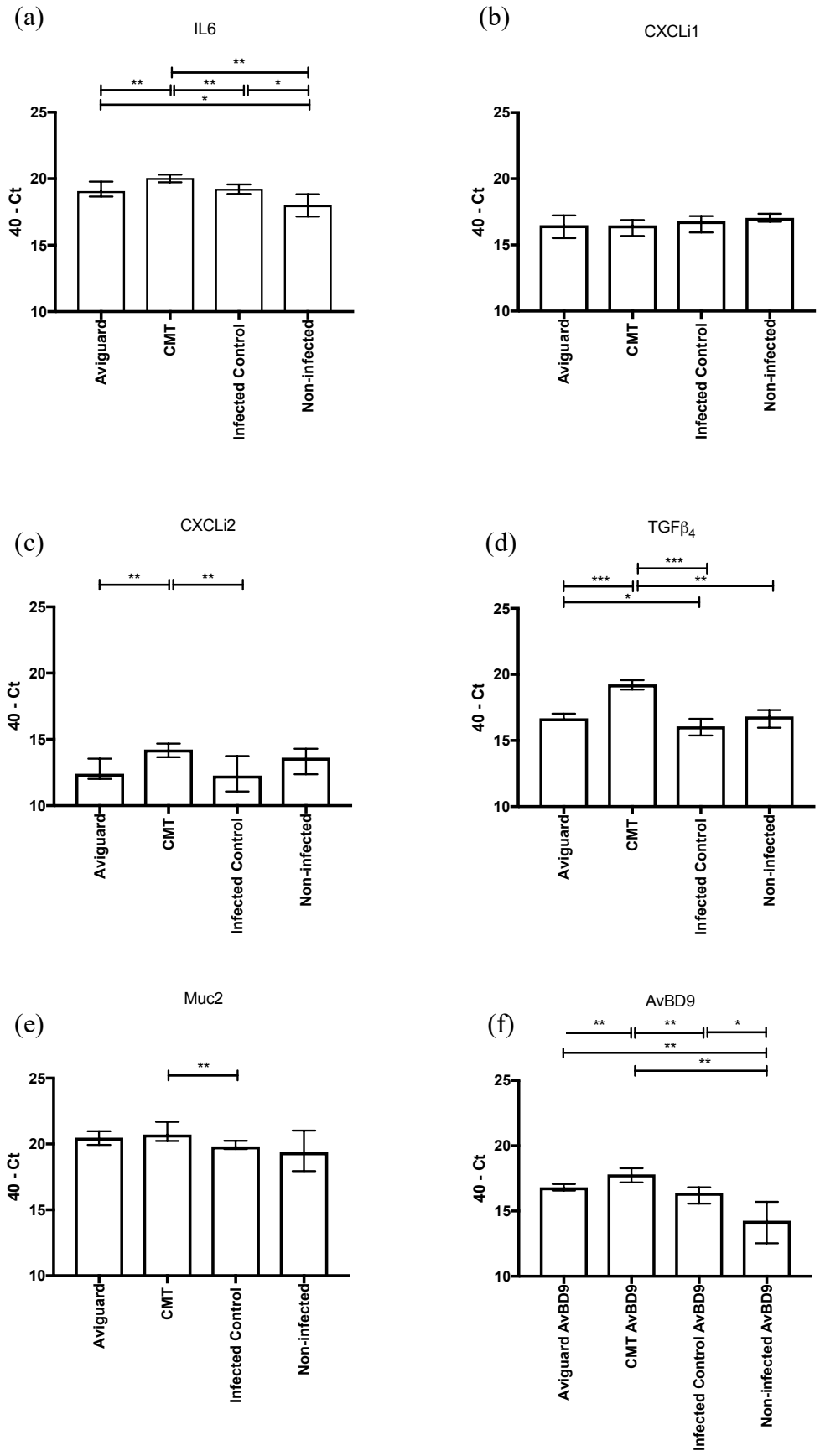


Figure 59. Expression of (a) IL-6, (b) CXCLi1, (c) CXCLi2, (d) TGFb4, (e) MUC2 and (f) AvBD9 genes given as 40 – C_t values following qRT-PCR analysis of RNA extracts from 8E11 cells challenged with *C. jejuni* M1 over 4 hours. Data is represented as median and respective IQR values. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p<0.05, **p<0.01, ***p<0.001. For all treatment groups, n=10; for non-infected control groups n=6.

Salmonella Typhimurium *4/74*

For cell invasion protocols modelling 4-hour infection with S. Typhimurium 4/74, as described for IL-1ß and IL-10, I was unable to attain reliable RT-qPCR reaction data using the chemokine ligand CXCLi2 with this data not being presented here (Figure 62 & Figure 63). Data regarding IL-6, TGFß$_4$ and CXCLi2 transcription all followed a similar trend in upregulation of expression compared to cells not challenged with S. Typhimurium ($p < 0.05$). Little variation in the degree of upregulation of the IL-6 and TGFß$_4$ cytokines between challenged treatment groups, however CMT cell treatment significantly reduced the upregulation of the chemokine CXCLi1 compared to both Aviguard® and infected control cells ($p < 0.05$).

MUC2 expression was significantly increased in all infected treatment groups compared to non-S. Typhimurium challenged cells ($p < 0.05$), with Aviguard® treated cells showing highest upregulation of 1.69 - fold. Interestingly, although infection had limited influence on AvBD9 expression for control and Aviguard® treated cell groups, a downregulation trend was observed for CMT treated cells of 0.06-fold compared to non-infected cells ($p = 0.0646$). Statistical analysis parameters and *p* - values for all treatment group comparisons are provided in Table 24.

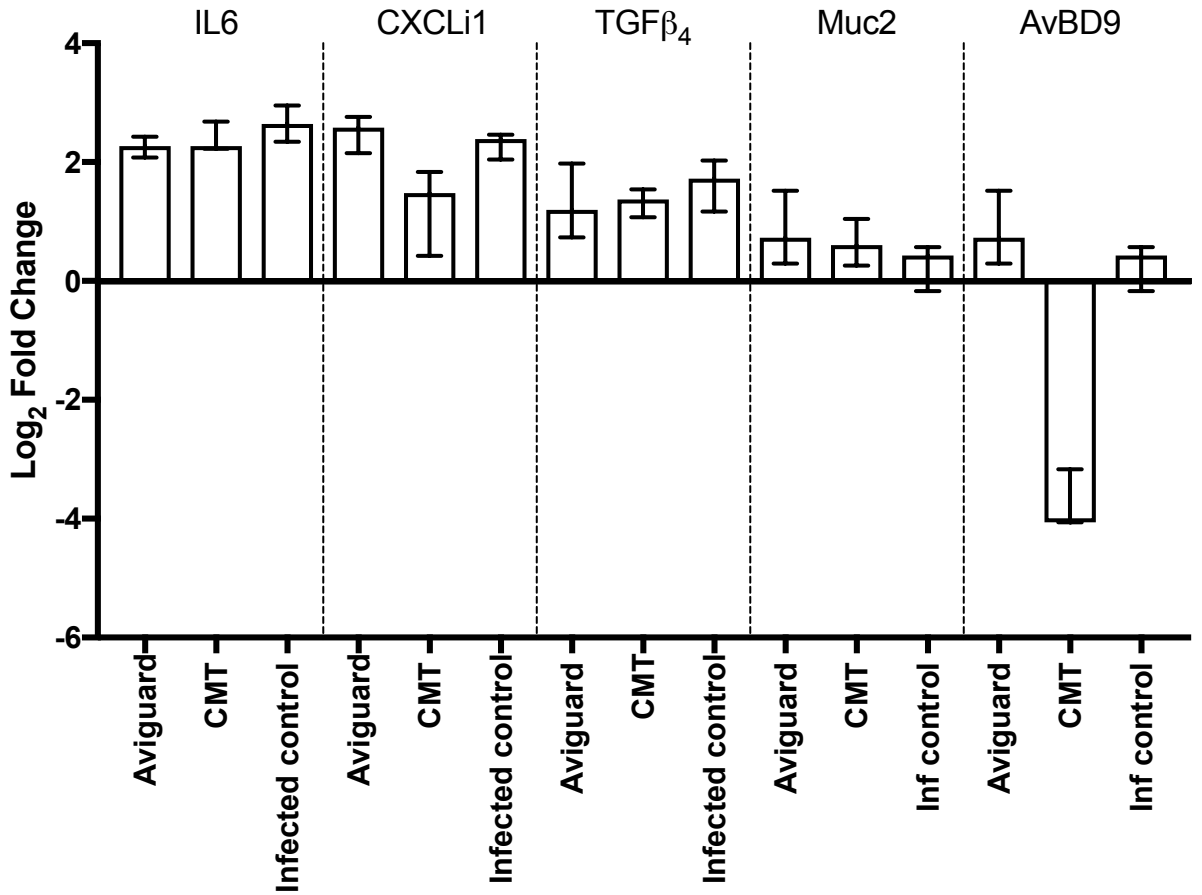


Figure 60. Log_2 relative expression of IL-6, CXCLi1, TGFß4, MUC2 and AvBD9 following RNA extraction from 8E11 cells challenged with S. Typhimurium 4/74 over 4 hours compared to non-challenged cells. Data is represented as median values with respective IQR. For all treatment groups, n=10; for non-infected control groups n=6.

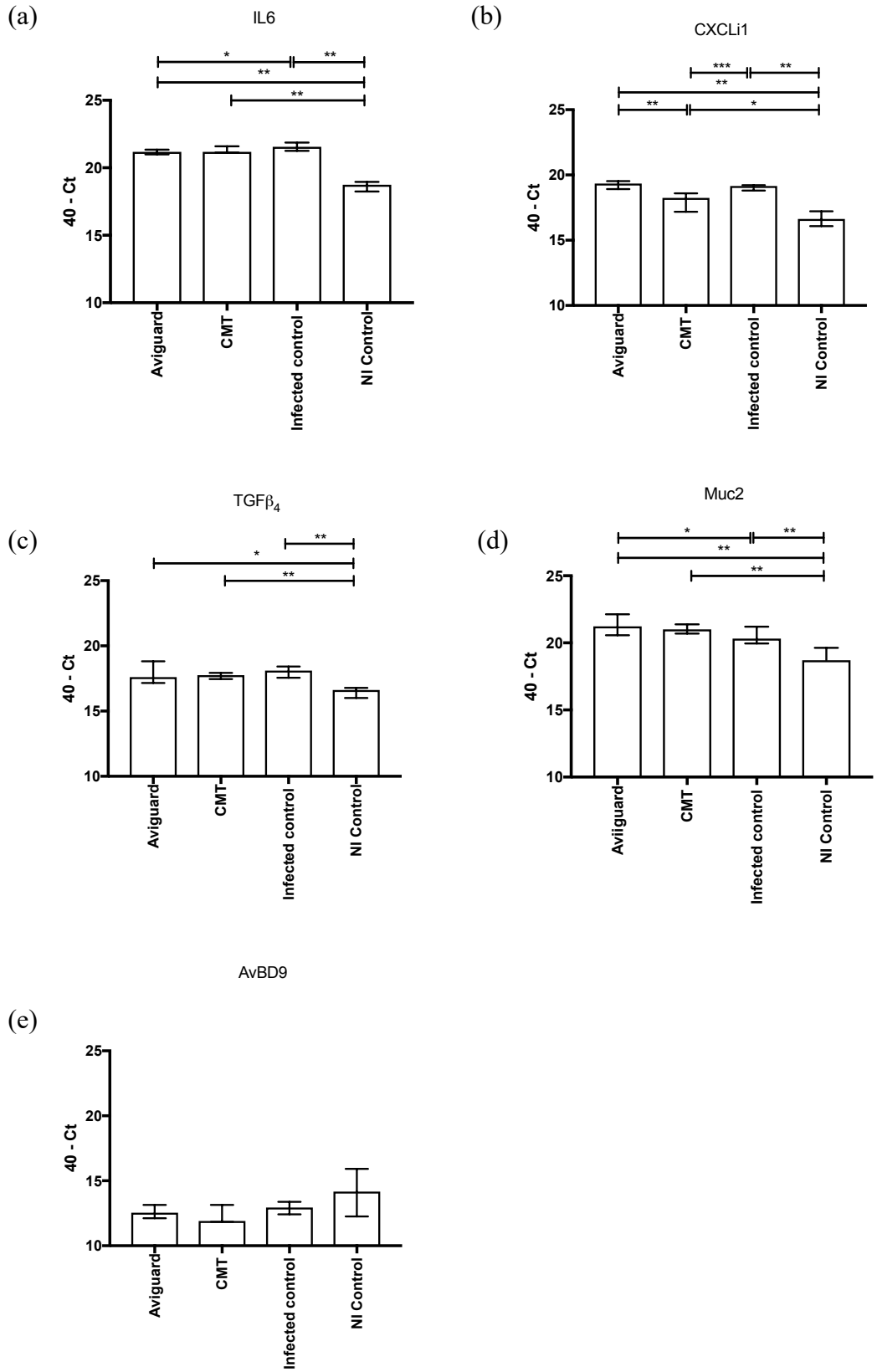


Figure 61. Expression of (a) IL-6, (b) CXCLi1, (c) TGFß$_4$, (d) MUC2 and (e) AvBD9 genes given as 40 – C_t values following qRT-PCR analysis of RNA extracts from 8E11 cells challenged with S. Typhimurium 4/74 over 4 hours. Data is represented as median and respective IQR values. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p<0.05, **p<0.01, ***p<0.001. For all treatment groups, n=10; for non-infected control groups n=6.

Table 24. Statistical significance values given for genes of interest following modified GPA protocols using 8E11 cell lines. Results depict incubation of 4-hours with specific challenge strain.

Bacterial challenge	Treatment group	Statistical test used	Group comparison	IL-6	CXCLi1	CXCLi2	$TGFB_4$	MUC2	AVBD9
C. jejuni M1	CMT	Mann Whitney-U	Aviguard®	0.007	0.8785	0.0012	0.0002	0.3823	0.003
			Infected Control	0.0019	0.4418	0.0047	0.0002	0.007	0.0019
			NI Control	0.0016	0.0932	0.1709	0.0016	0.0932	0.0016
	Aviguard®		Infected Control	0.7209	0.6454	0.7209	0.0379	0.069	0.0881
			NI Control	0.0451	0.1826	0.1274	0.9433	0.1709	0.0016
	Infected Control		NI Control	0.0109	0.4351	0.1709	0.1709	0.2844	0.0109
S.* Typhimurium *4/74	CMT	Mann Whitney-U	Aviguard®	0.3423	0.0011	n/a	0.5737	0.9329	0.3671
			Infected Control	0.2455	0.2345	n/a	0.0006	0.1049	0.0611
			NI Control	0.004	0.004	n/a	0.0283	0.004	0.0646
	Aviguard®		Infected Control	0.026	0.7984	n/a	0.3823	0.0463	0.2345
			NI Control	0.004	0.0162	n/a	0.004	0.004	0.2828
	Infected Control		NI Control	0.004	0.0283	n/a	0.004	0.004	0.4606

GENE EXPRESSION BY $2^{-\Delta\Delta CT}$ RT-qPCR: 24-HOUR CHALLENGE

As described for 4-hour challenge models already described, influence of CMT or Aviguard treatment was investigated for ability to influence inducible immune gene expression. Expression of IL-1β, IL-6, IL-10, TGFβ$_4$, CXCLi1, CXCLi2, MUC2 and AvBD9 mRNA was measured following RNA extraction from 8E11 cell lines at 24 hours post infection with either *C. jejuni* or S. Typhimurium (Figure 40). For both invasion models of this challenge duration, I was unable to attain reliable RT-qPCR reaction data using IL-1β, IL-10 and CXCLi1 primers and as such, the data presented within this chapter will exclude expression values for these data sets. All data presented was first assessed for normality of distribution using D'Agostino & Pearson analysis. Since all data did not adhere to a normal distribution ($p < 0.05$), description will be based upon median values and their respective IQR according to Mann-Whitney-U comparison-based analysis with a significance threshold of $p < 0.05$.

C. jejuni M1

For both cytokine transcripts showing reliable expression patterns (IL-6 and TGFβ$_4$), expression was significantly upregulated in all *C. jejuni* challenged groups compared to non-infected cell replicates ($p < 0.05$). For both IL-6 and TGFβ$_4$ gene transcripts, cells treated with Aviguard® filtrate showed highest expression of all *C. jejuni* challenged treatment groups ($p < 0.05$), with respective 4.98 - fold and 6.00 - fold increases compared to non-infected cells (Figure 63 & 64).

Expression of the chemokine ligand CXCLi2 was downregulated in all cell replicates challenged with *C. jejuni* compared to non-challenged cell replicates. Treatment of cells with CMT filtrate was correlated with strongest downregulation of CXCLi2 compared to non- *C. jejuni* challenged cells ($p = 0.0019$), with expression also significantly lower than both other *C. jejuni* challenged treatment groups ($p < 0.05$). Expression of MUC2 was not influenced by either *C. jejuni* challenge or specific treatment group within the 24-hour infection model used ($p > 0.05$). Conversely, AvBD9 was significantly upregulated in all cells challenged with *C. jejuni* compared to non-challenged cells ($p < 0.05$). Pre-treatment of cells with CMT resulted in a lower expression of AvBD9 transcripts compared to *C. jejuni* infected control and Aviguard® treated cell groups ($p < 0.05$). Statistical analysis parameters and p - values for all treatment group comparisons are provided in Table 25.

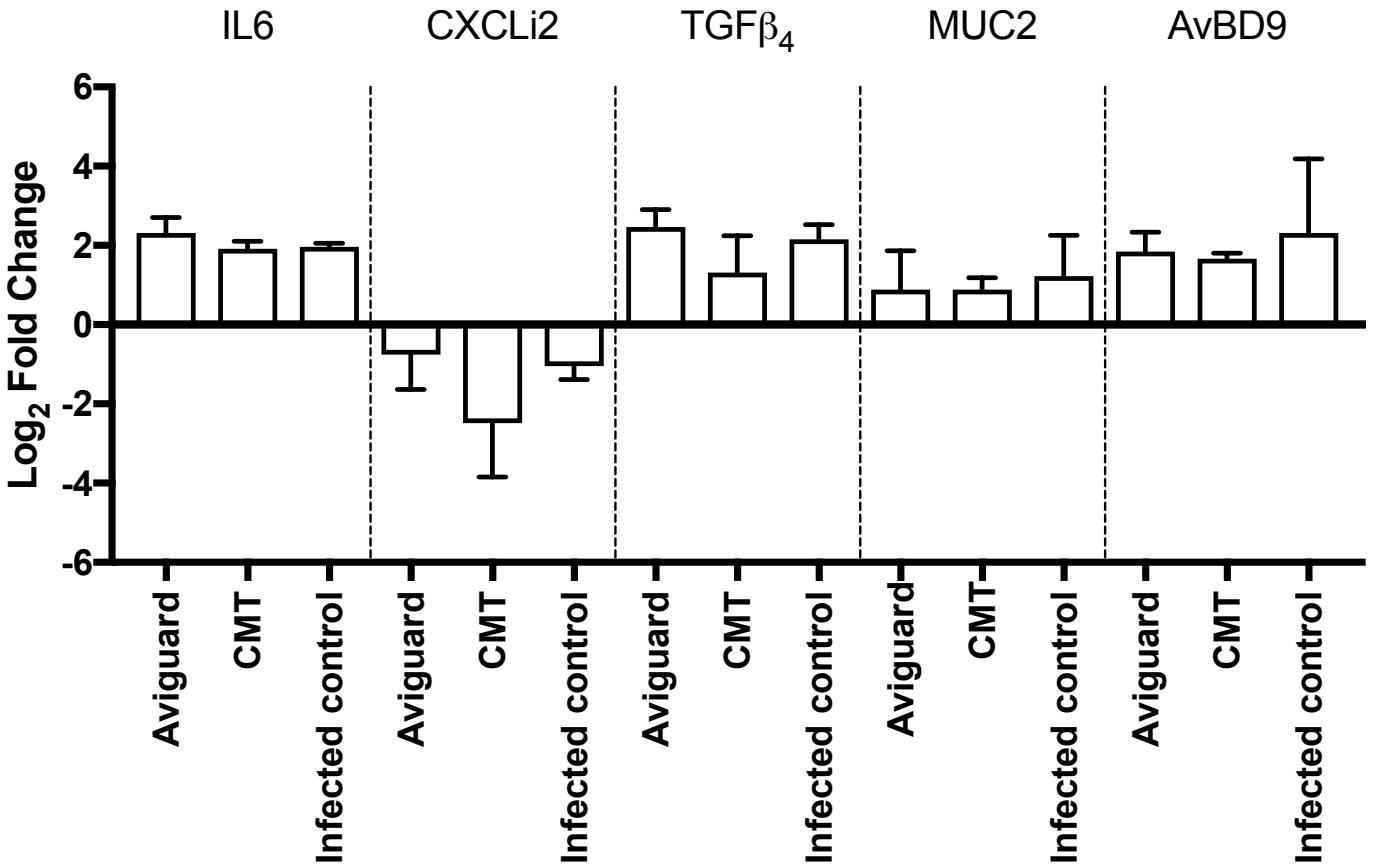


Figure 62. Log_2 relative expression of IL-6, CXCLi2, TGFß4, MUC2 and AvBD9 following RNA extraction from 8E11 cells challenged with *C. jejuni* M1 over 24 hours compared to non-challenged cells. Data is represented as median values with respective IQR. For all treatment groups, n=10; for non-infected control groups n=6.

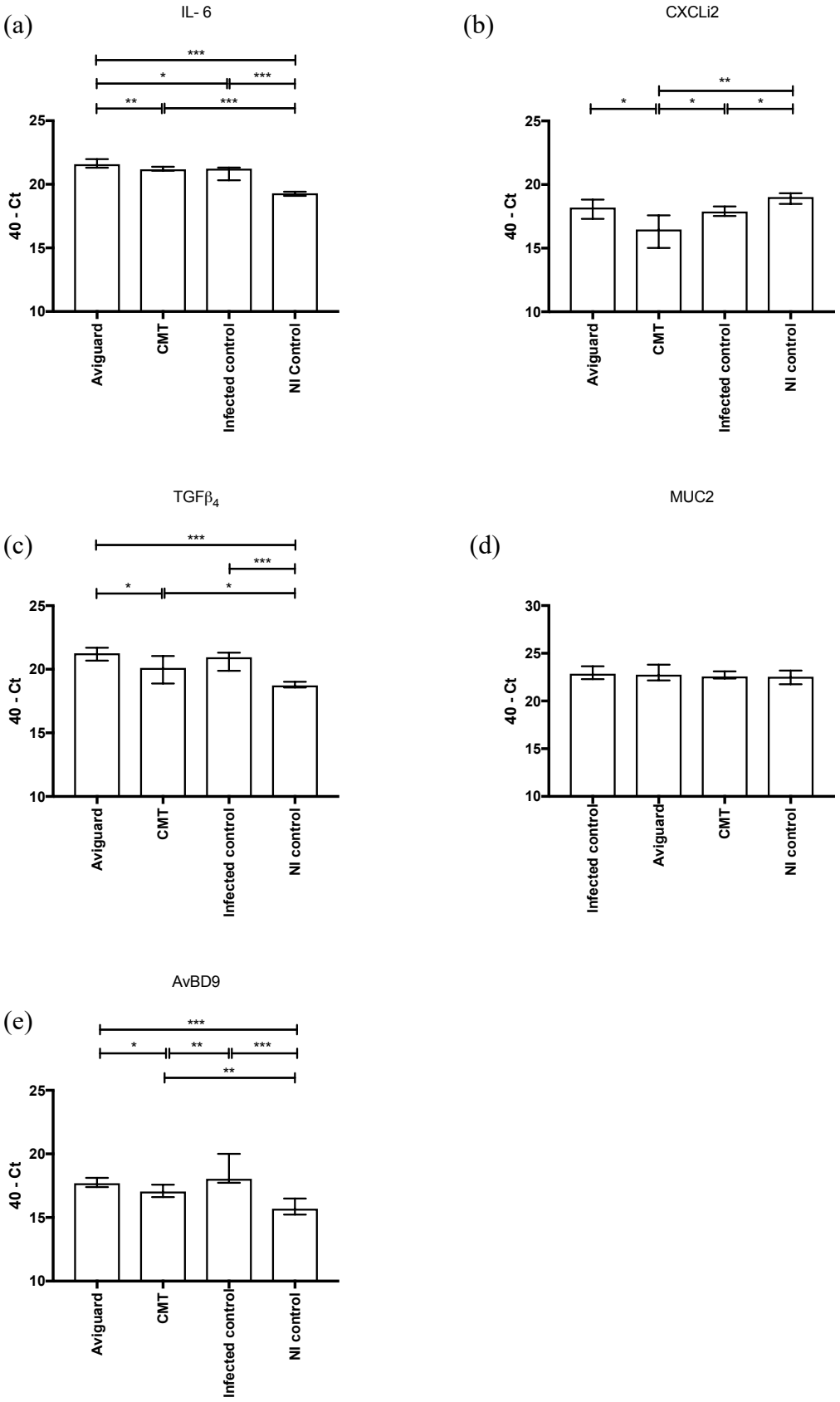


Figure 63. Expression of (a) IL-6, (b) CXCLi2, (c) TGFß4, (d) MUC2 and (e) AvBD9 genes given as 40 – C_t values following qRT-PCR analysis of RNA extracts from 8E11 cells challenged with S. Typhimurium 4/74 over 4 hours. Data is represented as median and respective IQR values. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p<0.05, **p<0.01, ***p<0.001. For all treatment groups, n=10; for non-infected control groups n=6.

S. Typhimurium *4/74*

Expression of IL-6 and CXCLi2 transcripts in all cells challenged with S. Typhimurium 4/74 was upregulated across all treatment groups compared to that of non-challenged cell replicates ($p < 0.05$), with CMT treated cells showing greatest increase at 12.94 - fold and 9.83 - fold respectively (Figure 65 & 66). While TGFß$_4$ expression was upregulated in infected control cell replicates ($p < 0.05$) but downregulated in both Aviguard® treated and CMT treated cell groups ($p < 0.05$).

S. Typhimurium challenge over 24 - hours resulted in downregulation of MUC2 expression for all cell replicates compared to non-infected cells. Pre-treatment of cells with CMT filtrate resulted in significantly lower expression of MUC2 transcripts compared to both other S. Typhimurium challenged treatment groups ($p < 0.05$). Little observable change in expression was observed for AvBD9 mRNa transcripts for both Aviguard® and CMT treated cells compared cells not challenged with S. Typhimurium with changes of 1.58 - fold and 1.39 - fold respectively. Untreated infected control cells showed significantly higher AvBD9 expression compared to unchallenged alongside CMT and Aviguard® treated cells ($p < 0.05$). Statistical analysis parameters and *p* - values for all treatment group comparisons are provided in Table 25.

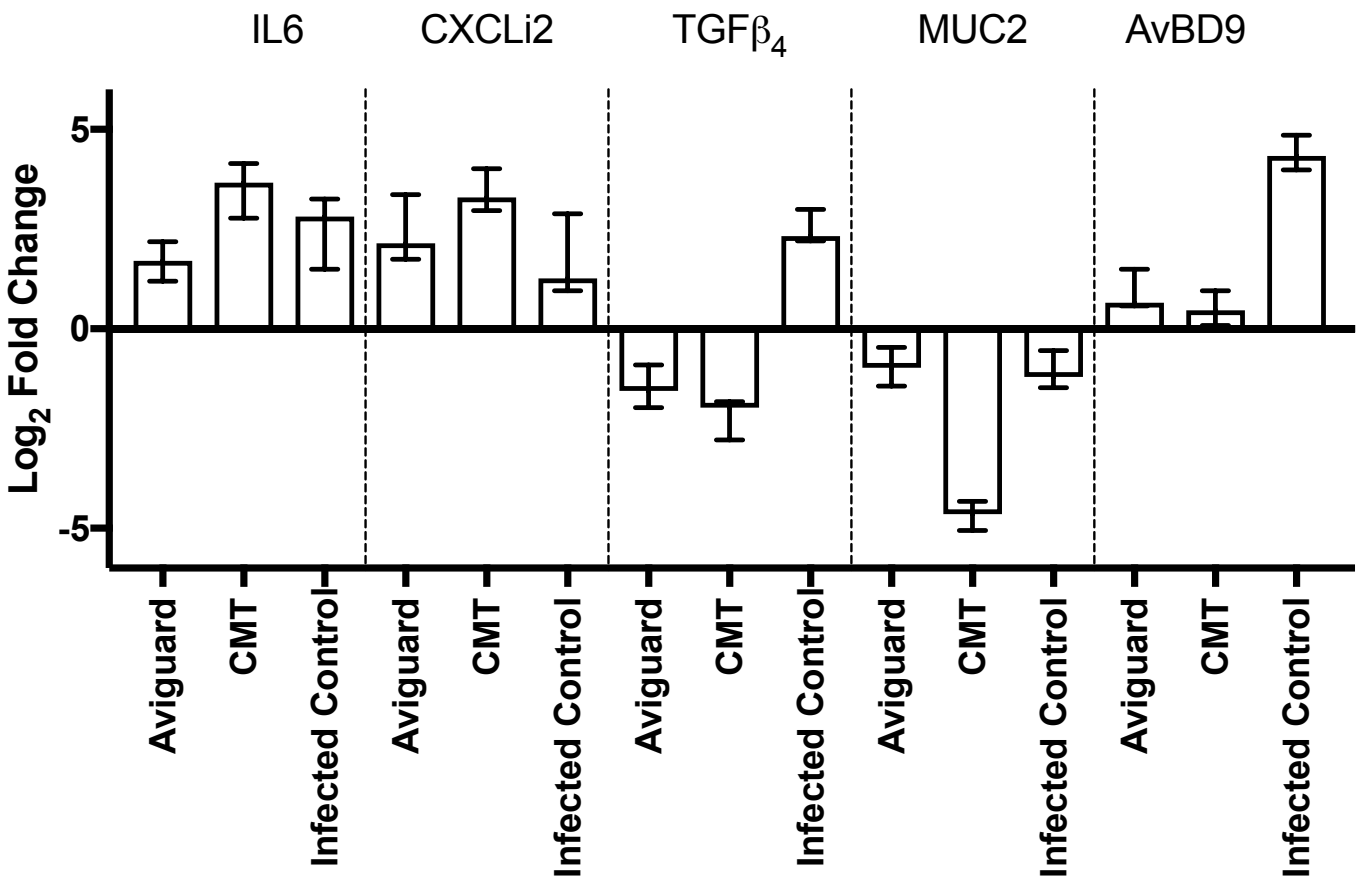


Figure 64. Log_2 relative expression of IL-6, CXCLi2, TGFß4, MUC2 and AvBD9 following RNA extraction from 8E11 cells challenged with S. Typhimurium 4/74 over 24-hours compared to non-challenged cells. Data is represented as median values with respective IQR. For all treatment groups, n=10; for non-infected control groups n=6.

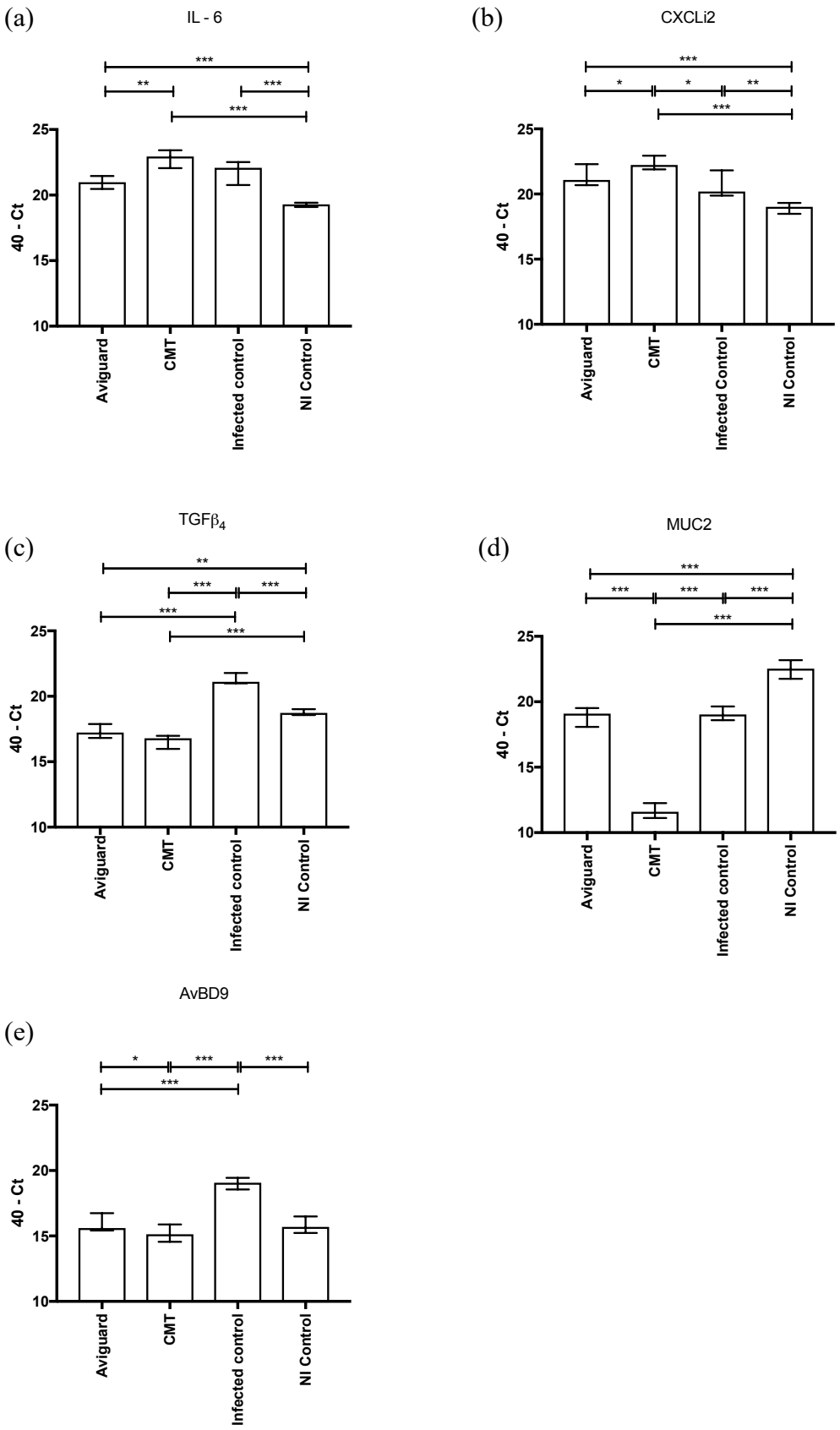


Figure 65. Expression of (a) IL-6, (b) CXCLi2, (c) TGFß$_4$, (d) MUC2 and (e) AvBD9 genes given as 40 – C_t values following qRT-PCR analysis of RNA extracts from 8E11 cells challenged with S. typhimurium 4/74 over 24-hours. Data is represented as median and respective IQR values. Statistical significance was determined using Mann Whitney-U analysis with levels of significance given as *p<0.05, **p<0.01, ***p<0.001. For all treatment groups, n=10; for non-infected control groups n=6.

Table 25. Statistical significance values given for genes of interest following modified GPA protocols using 8E11 cell lines. Results depict incubation of 24 hours with specific challenge strain.

Bacterial challenge	Treatment group	Statistical test used	Group comparison	IL-6	CXCLI2	TGFß$_4$	MUC2	AVBD9
C. jejuni M1	CMT	Mann Whitney-U	Aviguard®	0.0095	0.0499	0.0499	0.8575	0.0104
			Infected Control	0.9383	0.0281	0.2345	0.8177	0.0042
			NI Control	0.0002	0.0019	0.0379	0.7776	0.007
	Aviguard®		Infected Control	0.0162	0.8785	0.2786	0.8553	0.2238
			NI Control	0.0002	0.065	0.0002	0.4586	0.0002
	Infected Control		NI Control	0.0002	0.0148	0.0003	0.4244	0.0003
S.* Typhimurium *4/74	CMT	Mann Whitney-U	Aviguard®	0.003	0.0379	0.083	0.0002	0.0301
			Infected Control	0.083	0.0148	0.0003	0.0002	0.0002
			NI Control	0.0002	0.0002	0.0002	0.0002	0.2786
	Aviguard®		Infected Control	0.0876	0.1949	0.0003	0.8785	0.0002
			NI Control	0.0006	0.0002	0.003	0.0002	0.5737
	Infected Control		NI Control	0.0002	0.0047	0.0003	0.0002	0.0002

DISCUSSION

The modern broiler chicken offers one of the most efficient production systems available within food animal systems, being able to convert 6.37 kg of feed into a 3.48 kg weight gain in only 49 days (Oakley et al., 2014). While this phenomenon must be attributed to a multitude of concomitant factors, largely encompassing genetic background, the importance of the gastrointestinal microbiota is being increasingly recognised. It is through previous work, discussed in Chapter 3, that we have begun to highlight the efficacious potential of CMT administration in reducing broiler susceptibility to *Campylobacter jejuni* infection. Using similar approaches to those before, this study investigated CMT and a commonly used avian CE product for their antimicrobial activity against *C. jejuni*. Similar to trends previously described, prophylactic CMT administration was associated with significant disruption of *C. jejuni* colonisation as seen in both Internal and External control groups. Conversely, treatment of chicks with a commercial CE product, Aviguard®, had no observable influence on *C. jejuni* flock transmission and only a 1.05 - fold decrease in final caecal *C. jejuni* burden compared to infected control treatment groups. Such results provide additional evidence that CMT treatment possibly acts an effective prophylactic therapy to reduce *C. jejuni* colonisation of the commercial broiler chicken. It is, however, important to note the significant reduction in body weight potentially associated with such treatment compared to those treated with Aviguard® preparation. With unrelenting drive to attain high broiler body weights within the poultry industry, secondary impacts such as those associated with body weight might significantly influence the feasibility of such a product commercially. Increased weight gain and improvements in feed conversion efficiency have both been reported following probiotic and/or prebiotic supplementation of broiler chickens feed or water (Utami and Wahyono, 2018). Increased digestive enzyme production often associated high populations of bacteria such as *Lactobacillus spp*. alongside potential alterations to intestinal morphology that increase intestinal villi length, increasing surface area for absorption could provide reasoning for this. It remains unclear form this work why such differences in body weight were observed between Aviguard® and CMT treated birds, however it is imperative that future work explores this correlation. Such work would perhaps gain more useful insight by utilizing 'weight gain' measurements as oppose to sole use of a final weight measurement as used here.

At a point in time where more is understood about the mechanistic actions of many of our therapeutic interventions, microbial intervention strategies such as CE products and FMT remain oddly unsophisticated (Schneitz & Hakkinen, 2016). The introduction of what is

claimed to be a 'healthy' or 'balanced' gut flora into newly hatched broiler chicks with commercial CE products has been well documented over recent years (Khoruts, 2018). Since the early work of Nurmi and Rantala in 1973, modulation of the microbiota with CE products has become an established research area within published literature. Generally, much of the therapeutic potential described for CE cultures within poultry is centered on limitation of *Salmonella* infection, being confirmed across multiple studies (Zhang et al., 2007). This being said, extension of this research to incorporate *C. jejuni* control within the broiler chicken has been explored, with both defined and undefined cultures showing limited success (Chen & Stern 2001; Stern et al. 2001; Mead 2000). Although published reports of the effectiveness of Aviguard® in particular are scarce and relatively inconsistent, Nakamura et al. (2002) has gone some way in emphasizing its ability to provide prophylactic control over subsequent *Salmonella* spp. colonisation.

Minimal research is currently available detailing the efficacy of therapeutic administration of CMT to avian species to reduce susceptibility of disease. Of particular note, is research presented by Hofacre et al. (2000) comparing the efficacy of Aviguard® or fresh turkey caecal material in reducing *Salmonella* colonisation in turkeys. Reminiscent of the trends in colonisation seen within our own trial, administration of fresh caecal material (CMT) was significantly more protective compared to Aviguard® administration (Hofacre et al., 2000).

While the competitive exclusion mechanisms likely to be a fundamental factor associated with both Aviguard® and CMT products will be discussed in Chapter 5, here we assess their modulation of the immune system. Through our study, we tested the ability of Aviguard® or CMT to either modulate or inhibit the invasion of *Campylobacter* or *Salmonella* into of avian intestinal cells. Bacterial challenge strains were incubated with an avian intestinal monolayer alongside each therapeutic treatment (CMT or Aviguard®) and the invasion of challenge strain assessed.

Bacterial challenge status and prophylactic treatment group resulted in differential expression of both cytokine and chemokine parameters examined. While the data presented for both 4- and 24-hour represents a relatively small dataset, it provides a valuable basis for how CMT or Aviguard® therapies may demonstrate up- or downregulation of cytokines and chemokines post-challenge. From the results attained, it was observed that the pro-inflammatory cytokine IL-6 was upregulated in all cell models following challenge with either of the strains examined.

IL-6 is a cytokine usually indicative of acute phase protein synthesis and is critical in modulating the transition from innate to acquired immunity (Kaiser et al., 2000). Expression of CXCLi1 and CXCLi2 homologues following *Salmonella* infection exhibited trends toward early and sustained upregulation. However, with these chemokines assessed at only one time-point each – 4 hours and 24 hours post-infection respectively, biologically reliable conclusions cannot be formed. This tentative tendency toward upregulation following *Salmonella* infection has previously been described by Salisbury et al (2014) in tissues of the chicken gastrointestinal system following infection with *Salmonella* Virchow. Similar trends CXCLi2 response were not observed for *C. jejuni* challenge models. Treatment of 8E11 cells with CMT filtrate stimulated an upregulation of CXCLi2 expression 4 hours post *C. jejuni* challenge, being downregulated in both Aviguard® treated and infected control cells. At 24 hours post *C. jejuni* challenge, CMT treated cells conversely showed significant CXCLi2 downregulation compared to all other treatment groups. While the full complement of roles played by CXCLi2 in the chicken immune response to bacterial infection is yet to be elucidated, known chemotactic influence for monocytes and lymphocytes have been highlighted (Larson et al., 2008). The initial CXCLi2 upregulation seen within CMT treated enterocytes, alongside gradual upregulation of IL-6 appears indicative of rapid initiation of an inflammatory response particularly associated with CMT treatment. Work by Larson et al. 2009 has provided previous link between infection of chicken cell lines *in vitro* with *C. jejuni* and increasing chemokine ligand (CXCLi1 and CXCLi2) expression. John et al., (2017) provides extension to this by not only confirming the importance in CXCLi1 and CXCLi2 in early inflammatory response to a number of *C. jejuni* strains within a chicken epithelial cell line but highlighting how the response of homologue CXCLi2 may be significantly higher than that of CXCLi1 in this infection model.

Interestingly, the rapid induction of CXCLi chemokines during early (4 hours post-infection) *S. Typhimurium* infection was accompanied by an upregulated $TGF\beta_4$, anti-inflammatory response. By 24-hours post infection, control cells sustained such upregulation, while both Aviguard® and CMT treated cells showed significant $TGF\beta_4$ downregulation. $TGF\beta_4$, upregulation was only observed for CMT treated cells within the 4-hour post-infection *C. jejuni* model. By 24-hours post *C. jejuni* challenge, this $TGF\beta_4$ upregulation was present across all challenged treatment groups. It could be speculated that the initial surge in pro-inflammatory IL-6, but more importantly, CXCLi2 in CMT treated cell groups stimulated the transcription of the anti-inflammatory $TGF\beta_4$ as a compensatory mechanism resulting in subsequent pro-

inflammatory regulation. This expression profile proposed for TGFβ$_4$ has previously been described by Brisbin et al. (2010) who described TGFβ$_4$ expression and its involvement in maintaining intestinal homeostasis in the chicken reducing production of proinflammatory cytokines.

Using AvBD9 and MUC2 as markers of endogenous antimicrobial peptide (AMP) and glycoprotein response respectively following both microflora filtrate treatment and/or bacterial challenge provided further insight into how these therapies may influence innate immune response. Therapeutic treatment or challenge status showed little significant influence on MUC2 expression from enteric cells following *C. jejuni* challenge at both tested time-points. Conversely, AvBD9 was significantly upregulated in all treatment groups, most notably CMT treated, compared to non-challenged cells at 4-hours post infection. Transcriptional profiles of various β-defensins have been associated with bactericidal activity against multiple enteric pathogens, including *Salmonella* Typhimurium both *in vivo* and *in-vitro* (Garcia et al., 2018; Hong et al., 2012). More recently, Garcia et al., (2018) assessed the expression of multiple AvBD genes (AvBD1, AvBD6, AvBD8, 1 AvBD10, AvBD11, AvBD12 and AvBD13) in the ileal and caecal tissues of broiler chickens experimentally infected with doses of *C. jejuni*, with all being significantly upregulated following challenge at 10^6 cfu. This relationship was observed to be dose dependent, with higher doses showing no such expression (Garcia et al., 2018). This role in innate host defense against food borne pathogens and an apparent upregulation in expression within CMT treated cells may provide a basis of understanding the reduced bacterial invasion recorded here using gentamicin protection assay techniques. With potent induction of AvBD9 provided by pro-inflammatory cytokines such as IL-6, such AMP's may be being promoted and utilised as effector molecules in the very early stages post challenge (Hong et al., 2012). Such response could perpetuate further pro-inflammatory cytokine expression and reduced *C. jejuni* systemic invasion. Supplementary to this, while negligible following only 4-hour challenge strain incubation, both *C. jejuni* and S. Typhimurium were able to induce significant increases in cell nitric oxide release by 24 hours post-infection. Produced by a wide variety of cells, nitric oxide acts plays a crucial role in host defense against infection through the gastrointestinal system (Singh et al., 2012). Contradictory to initial expectation, *C. jejuni* challenge showed little impact on MUC2 expression using avian cell models. Interaction of *C. jejuni* with the avian intestinal mucosal liming is thought to be crucial to its infection biology due to its lack of invasive ability (Smith et al., 2008). Having been previously linked to attenuation of *C. jejuni* invasion, our work

implies that alterations in MUC2 expression was not underlying the variation in invasiveness of *C. jejuni* according to specific treatment group within this intestinal cell line. *Salmonella* infection appeared to induce different MUC2 expression characteristics, exhibiting early upregulation 4 hours post infection compared followed by significant downregulation, with this being particularly associated with CMT treatment. While this work provides notable insight into broiler chicken intestinal immune response to bacterial challenge and how this might be manipulated through administration of probiotic filtrate, there exist a number of limitations. An important limitation of the *in vitro* models described here are that they are monolayer cellular tissues that harbor minimal biological complexity or immunological potential offered within the natural *in vivo* system. Additionally, while known as an intestinal epithelial cell line, the 8E11 cells used within this experimental work show little in the way of published experimental use and as such, understanding of cellular limits in transcriptional response following challenge is limited. Assessing the expression profiles associated biological tissue samples collected from *in vivo* tissue samples may provide more biologically relevant answers to gaps in our current knowledge. The high variation in control cell technical replicate may also call the biological relevance of these findings into question. While the importance of such a limitation cannot be underestimated, and may prevent reliable intergroup comparisons, the results presented may pose interesting theoretical understanding that can underly further experimental analysis.

Work by Sahin et al. (2003) has previously shown a weak IgM and IgA response to *Campylobacter* infection within the chicken during the first two weeks of infection. While this appears to be the case regarding IgY response within our study, all study treatment groups experimentally infected showed significant increases in serum IgM titres by 14 d.p.i. Sahin et al (2003) goes on to state that IgY response is often significantly preceded by that of IgA and IgM, occurring much later in *C. jejuni* infection, potentially even that of systemic biology. This may provide explanation as to why some chickens underwent systemic *C. jejuni* infection but lacked corresponding IgY response. IgM is usually associated with primary antibody response to infection, being the initial immunoglobulin isotype secreted during infection (Schroeder & Cavacini, 2010). CMT treatment showed significant influence on increases *Campylobacter*-specific serum IgA by this time point, with a lack of IgA response observed across all other study treatment groups. As such, CMT treatment may influence early stimulation of the avian immune response, leading to rapid induction of IgM secretion and subsequently, an earlier induction of IgA. While of interest, it must be noted that work by Lacharme-Lora et al. (2017)

using bursectomy techniques in the broiler chicken illustrates how although present, increases in antibody titre during these early stages of life do not correlate with decreases in *C. jejuni* colonisation of the caeca. The presence of Immunoglobulin levels of all classes in non-infected chickens also confirms that observed measure of absorbance cannot be fully indicative of response to an active *C. jejuni* infection, or in fact, CMT treatment.

While much of the influence of CE cultures and CMT are thought to be derived from direct microbe interactions once established within the intestinal tract, here we show that this understanding can be developed. Using only sterile filtrate transfer of both Aviguard® and CMT we were able to alter the pathogenic potential of both *Campylobacter* and S*almonella* challenge strains using an *in-vitro* model. Such an effect provides further indication that additional elements of both Aviguard® and CMT, such as microbial metabolites can also be effective. Multiple published findings have established a strong understanding of particular bacterial metabolites, particularly SCFAs such as acetate, propionate, and butyrate are crucial control mechanisms through which microbial components of the gut can modulate the host immune system (Hong et al., 2019). Nagpal et al. (2018) have made further indication that an increase in taxonomic diversity within the microbiome will result in increased production of SCFAs, with a diverse range of microbial communities all able to produce such metabolites, including *Bacteroides, Ruminococcaceae, Lachnospiraceae*. Such work further states how potential microbe-microbe 'cross-feeding' behaviors may underlie this relationship (Nagpal et al., 2018). With FMT-, and similarly CMT- derived samples known to possess increased composition of bacterial commensals including SCFA-producing taxa such as *Ruminococcaceae* compared to more modified probiotic cultures, it may be sensible to infer that the CMT material used within this work derives potential for more SCFA production compared to that of Aviguard® based on a higher natural taxonomic diversity.

Chapter Five: Effects of Caecal Microbiota Transplantation on the microbiota of broiler chickens

INTRODUCTION

The microbiome encompasses the entire collection of genetic compliment from a microbial community representing commensal, pathogenic, and symbiotic organisms co-existing within a specific host (Cisek & Binek, 2014). Although comprising a diverse taxonomic range of organisms including bacteria, fungi, archaea, protozoa and viruses, this interrelated microbial community should not simply be considered on the basis of a sum of its individual components, but more an interactive organ in its own right (Borody et al., 2013).

From the moment of hatch, the chicken microbiome begins to be established. It is this first exposure to microorganisms from the egg shell, hatchery environment and, over subsequent days, litter, water and feed within farm sheds that will ultimately determine the intestinal microbiota that develops in the first weeks of life (Schokker et al., 2014). Successive colonisation of the gastrointestinal tract (GIT) of chicks commonly undergoes two successional stages during the first week of life, developing from a community dominated by *Enterobacteriaceae* during days 1 – 3 post-hatch, to one dominated by *Firmicutes* from 7 days post-hatch (d.p.h) onward (Connerton et al., 2018). Adding complexity, the broiler GIT will undergo divisional temporal changes in microbiota development whereby intestinal segments differentiate in their microbial community ecology (Jurburg et al., 2019). Due to the nature of the commercial broiler industry, all individuals within a commercial broiler flock will invariably share consistent environmental influences and so will exhibit a somewhat common microbiome community at each point of development. For the purposes of this research, our focus will be solely on the caecal microbial community since this is the most intestinal section most densely populated with bacteria and is the microbial community most likely to encounter extended contact with *C. jejuni* following experimental infection (Newell & Fearnley, 2003).

Notably, Juburg et al (2019) has emphasised how caecal microbiota taxonomic diversity increases most during the first 7 days of life, further highlighting how bacterial succession during this period could be crucial in the development and stability of microbiomes in adult broiler chickens. It is during this time that bacterial succession of the microbiome is most extreme, exhibiting radial shifts in composition on a daily basis (Jurburg et al., 2019; Richards et al., 2019). Microbial function is intrinsically determined by the presence of specific taxa and even small numbers of certain microorganisms are able to exert significant impact on overall

microbial communities. Potentially undesirable shifts in microbial communities stemming from this may have substantial influence on host health and performance (Cisek & Binek, 2014). With the GIT presenting an interface whereby the avian microbiota is in direct contact with the host epithelial surface, these host-microbe interactions are heavily linked to intestinal development and maturation, immunomodulation, maintenance of intestinal homeostasis and contribution to host nutrition (Shang et al., 2018). Shang et al (2018) described how an imbalance in the normal microbial ecology of the GIT, or dysbiosis, can lead to sequential deterioration in function of the intestinal epithelium, poor nutrient digestibility, increased risk of bacterial translocation and subsequent inflammatory responses.

With poultry meat consumption showing no significant decrease worldwide, a continued drive for efficiency of production within the poultry industry exists (FAO 2019). While it remains undeniable that the microbial communities inhabiting the intestinal tract of all animals will have profound impact on the nutritional state of the host, the intensive nature of the poultry production makes this facet imperative (Ramakrishna, 2013). Consistently production of broiler chickens with a 'healthy' and stable microbiome will increase the production of host-utilisable elements within distal regions of the gut such as SCFAs and amino acids that all represent an energy source readily available for the bird (Oakley et al., 2014; Shang et al., 2018; Van Der Wielen et al., 2000). Dibner & Richards (2005) also highlight additional function of SCFAs in the stimulation of intestinal epithelial cells to rapidly proliferate, resulting in increased villi surface area for absorption (Oakley et al., 2014). Natural, endogenous production of multiple dietary vitamins by the healthy intestinal microbiota could also aid the common practice of dietary vitamin supplementation seen in broiler chicken production (Oakley et al., 2014).

Of perceivably greater importance, is the relationship between the development of a healthy chicken microbiome in the caecum and the reduced susceptibility of chicks to various enteric pathogens (Pan & Yu, 2014). Colonisation resistance, as described in work by Pickard et al. (2017) shows commensal gut symbionts forming an established microbial community that in turn, resists the invasion of non-native bacteria through a combination of potentially bactericidal and bacteriostatic mechanisms. Cisek & Binek (2014) have corroborated this theory in poultry, showing, in the absence of a normal caecal microbiota, chicks were appreciably more susceptible to opportunistic infection. In spite of the fact that a definitive mechanism responsible for this protection continues to be elusive, competitive exclusion (CE)

processes are widely heralded as the driving force, described in early work by Nurmi & Rantala (1973) in the protection of broiler chickens against *Salmonella* infection. As explained previously, ecologically, CE negates the ability of two species competing for the same limiting resource to coexist as a stable community (Mead, 2000; Schneitz, 2005). This competition within a particular niche may be in the form of physical competition for space, resources or direct confrontation between native and invading colonisers (Clavijo & Flórez, 2018). A microbiota undergoing dysbiosis represents a less stable community which, in turn, will be less able to compete with invading pathogens. In addition, although variation exists in exact microbiome composition between individual adult chickens, the acquired microbiome will work to develop the immune system, pertaining particularly to the mucus layer, epithelial monolayer and intestinal immune cells (Shang et al., 2018). Shifts in the microbiota community constitution can both beneficially optimise immune capability but, in the same respect, hamper its function (Broom & Kogut, 2018). It is undeniably clear that the intestinal epithelium and adjacent microbiota are crucial in understanding how gut health can be maintained and consequently, how any impairment may increase host susceptibility to disease (Awad et al., 2018).

Although a comprehensive understanding of avian microbiome structure and respective functionality is important in many aspects of commercial production, until recently, our ability to define this structure has been reliant on culture based microbial techniques (Arnold et al., 2016; Oakley et al. 2014). Recovery of an accurate representation of the avian gut microbiome through microbiological cultivation is inefficient and vastly inaccurate, and so the advances in next generation sequencing over recent years has opened new opportunity to assess the previously unculturable communities (Stanley et al., 2014). Early studies often utilised 16S rRNA sequencing techniques for such purposes (Zhu et al., 2002; Lu et al., 2003). Supported by both culture-based and 16S sequencing techniques is the understanding that the caecal microbiome is predominated by anaerobes, with fewer facilitative representatives (Lu et al., 2003). Shaping of the caecal microbiota during early successional development is thought to be particularly dependent upon environmental exposure and so compositional observations vary somewhat between experimental study (Richards et al., 2019). However, it is largely corroborated that the caecum is dominated by *Clostridiaceae, Bacteroidaceae, Lactobacillacea* and *Lachnospiraceae* families, alongside an experimentally enticing abundance of uncharacterised microbial taxa (Borda-mollina et al., 2018).

As highlighted in previous chapters, *C. jejuni* infection within the commercial broiler chicken is subject to a strong age-based delay, the lag-phase, with colonisation occurring predominantly at around 3 weeks of age (Newell, 2002). At this point of infection, *C. jejuni* colonisation is rapid and efficient, with the frequency of colonisation within a flock increasing from 5 to 95 % within as little as 6 days (Connerton et al., 2018). Work by Haag et al. (2012) has gone some way in demonstrating how the ability of *C. jejuni* to colonise within a mouse model is heavily dependent on the microbiome of the individual host, and after inducing significant microbial shifts, the mouse microbiome is heavily reliant on presence of *C. jejuni* infection. Sofka et al. (2015) noted that, although not statistically significant, *C. jejuni* infection of the chicken was associated with higher *Proteobacteria* and *Bacteroidetes* taxons as oppose to higher *Firmicutes* taxons in *C. jejuni* negative samples. Subsequent work by Sakaridis et al. (2018) into the association between the broiler microbiome and *Campylobacter* burden showed a somewhat contrary outcome, with higher *Firmicutes* composition concomitant with higher *Campylobacter* counts. Perhaps of most interest, is the evidential link between *Enterobacteriaceae* presence within chicken microbiome and the *C. jejuni* burden of the host (Bereswill et al., 2011; Sakaridis et al., 2018; Sofka et al., 2015). It remains largely undetermined how the colonisation of the broiler chicken with *C. jejuni* will ultimately impact the microbiota and which, if any, microbial communities may be important in reducing or prohibiting initial infection.

Here we aim to elucidate how the early (up to 7 d.p.h) microbiome of the broiler chicken can be manipulated through point-of-hatch inoculation with a commercial microflora preparation or a Caecal Microbiota Transplantation (CMT), and how this might influence subsequent *C. jejuni* infection.

MATERIALS AND METHODS

SAMPLE COLLECTION

Caecal samples used for DNA extraction and subsequent 16S rRNA sequencing were obtained from the experimental trial conducted in accordance with the complete experimental protocol ZIPP 54 and discussed in Chapter 4.

A total of 13 caecal samples (CMT n = 4; Aviguard® n = 3; Internal control n = 3; External control n=3) were aseptically collected from chicks 3 d.p.h and a further 40 caecal samples (10 samples per treatment group) were collected 7 d.p.h. Caecal samples were collected from both caecal sacs to create one pooled sample per bird. All samples were immediately snap-frozen post-collection in liquid nitrogen before storage at – 80°C for no longer than 2 weeks.

DNA EXTRACTION AND PREPARATION

Microbial community DNA was extracted from the collected caecal samples using the Qiagen QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the protocol for the 'Isolation of DNA from Stool for Pathogen Detection'. Since stool samples were frozen prior to extraction, a scalpel and spatula were used to scrape caecal material into a 2 ml sterile centrifuge tube on ice to a final sample weight of 180 – 220 mg. DNA extraction protocols were combined with an initial bead-beating step to improve microbial DNA yield. All samples were eluted into 200 µl DNase-free water (Qiagen, Hilden, Germany) as oppose to the stated Buffer ATE as required for further downstream analysis. Low DNA concentrations (of < 10 ng/µl) were obtained for some samples and so elutant was passed through the column filter twice as part of the final centrifugation step (Step 14). Samples were stored at – 20 °C until further processing. Opsonisation of DNA extraction according to our samples was performed by comparing three separate extraction kits for microbial DNA yield. Inclusion of InhibitEX ensured effective removal of PCR inhibitors from extracted samples. To account for the effects of contamination introduced through DNA extraction reagents and disposables, negative controls consisting of only nuclease-free water, with no sample added, were processed alongside our samples of interest. These were included on all agarose gel processing, being discounted form downstream 16S rRNA processing if no band was observed.

Subsequent to extraction, 1 µl of each DNA sample were comprehensively assessed for yield and quality using NanoDrop (ND-1000) Optical Density (OD) 260/230 and 260/280 ratios. Both ratios interpret DNA extract quality in relation to contamination and potential problems with the DNA extraction procedure used to obtain the sample. An OD 260/280 ratio of ~ 1.8 and an OD 260/230 ratio of ~ 2.0 – 2.2 were accepted as being optimal for good quality extracted DNA. To improve the yield and reduce contaminants for some extraction samples, DNA was precipitated in ethanol. For samples that required precipitation, 10 µl of 3M Sodium hydroxide (NaOH) (Sigma-Aldrich, Poole, UK) was added before a further addition of 275 µl of cold 100 % Ethanol. Samples were vortexed at maximum speed for 3 seconds before being incubated at – 80 °C for at least 1 hour. Following incubation, samples were thawed at room temperature (RT) and centrifuged at 12,000 x *g* for 30 minutes at room temperature. The DNA pellet was identified visibly, and any supernatant discarded. The DNA pellet was rinsed with 1 ml of 70 % Ethanol before being centrifuged at 12,000 x *g* for 15 minutes. The pellet was visibly identified, and the supernatant discarded. The remaining purified DNA pellet was air dried to remove remaining Ethanol before being rehydrated in 100 µl DNase free water. Samples were stored at – 20°C until further quality analysis.

After extraction and purification methods, DNA samples were assessed for quality and integrity. DNA were visualised using agarose gel electrophoresis using a 2 % (w/v) gel. The agarose gel was prepared by dissolving 1 g high pure low EEO agarose powder (Alpha Laboratories, Eastleigh, UK) in 100 mL 1 X Tris-acetate-EDTA (TAE) buffer (Promega, Southampton, UK). The agarose solution was heated until molten and all agarose powder was fully dissolved. After cooling of the molten agarose to below 55 °C, 5 µl of peqGREEN (2×10^{-5} µl/ml; Peqlab, Fareham,UK) was added and incorporated fully into the solution by swirling. The agarose was poured directly into a gel cassette before being allowed to set for 1 hour. DNA samples were prepared for loading by mixing 5 µl extracted sample DNA and 1 µl dye. Lambda *hind*III ladder (ThermoFisher Scientific, Loughborough, UK) was loaded into a pre-designated lane. 12 µl of Prepared DNA/loading buffer pertaining to individual samples were loaded into subsequent lanes. The loaded gel cassette was placed into an electrophoresis tank containing TAE buffer. The electrophoresis was run at 120 V for 30 minutes before DNA bands were visualised under ultraviolet transillumination using Uvitec software. Level of degradation was assessed through identification of more than one prominent band or a smear.

Purified DNA samples were also amplified using Polymerase Chain Reaction techniques using primers specific for the 16S rRNA gene (spanning the V4 region) (F: 5'-TGCCAGCMGCCGCGGTAA-3', R: 5'-GGACTACHVGGGTWTCTAAT-3') to confirm the production of visible gel bands of the expected size. PCR reactions were conducted using an Applied Biosystems 2720 thermo cycler (Thermofisher Scientific, Cheshire, UK) in 0.2 µl PCR tubes. PCR reactions contained 17 µl water, 5 µl 5x FIREPol® master mix ready to load with 7.5 mM $MgCl_2$ (Solis-Biodyne, Tartu, Estonia), 1 µl each of the forward and reverse primers, and 1 µl of genomic DNA. PCR was performed according to the following conditions: 95 °C for 5 minutes and 30 cycles of denaturing at 95 °C for 30 seconds, annealing at 55 °C for 45 seconds and elongation at 72 °C for 40 seconds, with a final extension of 72 °C for 5 minutes (Caporaso et al., 2012). Samples were subsequently run on a 2 % agarose gel as described above.

DNA was quantified with Qubit fluorometic analysis using a dsDNA High Sensitivity Assay (Invitrogen, Thermofisher Scientific, Loughborough, UK) was used, with a quantification range of 0.2 - 100 ng. DNA samples were submitted for V3/V4 hypervariable 16S rRNA gene amplification and Illumina MiSeq platform processing.

16S rRNA GENE SEQUENCING

Extracted DNA was sent for Illumina MiSeq sequencing of the V3/V4 hypervariable 16S rRNA gene at the Centre for Genomic Research (University of Liverpool). Briefly, primers used by Caporaso et al. (2010) were used to amplify the V4 region of 16S through a two-stage nested PCR;

F: 5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCCAGCGCCGCGGTAA3'
R: 5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACGGGTTCTAAT3'

This first round PCR reactions contained 5 µl of DNA with PCR conditions of 10 cycles set at 95°C for 20 seconds, 65°C for 15 seconds. 70°C for 30 seconds. This was then followed by a 72°C extension step for 5 minutes.

Primers were designed through incorporation of a recognition sequence to allow a secondary nested PCR process after purification with AMPure SPRI beads (Beckman Coulter, Indianapolis, US). This secondary PCR is performed largely for the incorporation of Illumina adapter sequences for sample sequencing on Illumina platforms in addition to the incorporation of

barcode sequences used for sample identification. The sequences used for forward and reverse priming of this PCR are illustrated below with the 8 base-pair (bp) barcode sequence being underlined.

N501F:5'AATGATACGGCGACCACCGAGATCTACAC*TAGATCGC*ACACTCTTTCCCTACACGACGCTC3'
N701 R : 5'CAAGCAGAAGACGGCATACGAGAT*TCGCCTTA*GTGACTGGAGTTCAGACGTGTGCTC3'

Creating an additional 15 cycles using the same conditions as previously described, a total of 35 cycles were performed. Samples were again purified using AMPure SPRO beads (Beckman Coulter, Indianapolis, US) before being quantified using Qubit fluorometic analysis with successful libraries proceeding to further processing.

Based on data from the Qubit and fragment analyser, and according to a Pippin Prep (Sage Science, Beverly, MA, US) size range of 300 – 600 bps, final libraries were pooled in equimolar quantities for Bioanalyser assessment. Using Illumina® Library quantification kits (KAPA, Wilmington, MA, US), qPCR was performed on a Roche Light Cycler® LC48011. All 20 µl qPCR reactions were performed in triplicate for each pooled library and prepared using 12 µl SYBR Green I Master Mix and 4 µl of the diluted pooled DNA. Thermal cycling conditions were set at 5 minutes at 95°C for denaturing, 35 cycles of 30 minutes of 95°C (denaturing) and 60°C for 45 seconds for annealing and extension followed by melt curve analysis reaching 95°C and subsequent cooling at 37°C.Diluted 0.1M NaOH was used to denture the final pool for 5 minutes before reaction termination by the addition of HT1 hybridization buffer with a final loading concentration of 11pM. All libraries were subsequently sequenced on an Illumina MiSeq platform with version 2 chemistry using sequencing by synthesis (SBS) technologies to generate 2 x 250 bp paired-end reads. CASAVA v1.8.2 was used to demultiplex all synthesised reads while Cutadapt v 1.2.1 (Martin, 2011) was used to remove any Illumina adapter sequences.

MICROBIOTA ANALYSIS

16S rRNA sequencing data processing was performed using the next-generation bioinformatics platform QIIME 2™ (2019.1) [Quantitative Insights Into Microbial Ecology v.2] (Bolyen et al., 2019). Microbial diversity within a treatment group was determined using alpha diversity parameters and diversity between treatment groups was determined using beta diversity parameters. The complete list of commands used can be found in Appendix 3 with a

brief overview provided in Figure 66. Forward and reverse Fastq raw sequencing data were imported into QIIME 2™ through creation of a manifest file. Created as a CSV (comma-separated text-file), the manifest file served to map sample identities with sequence data by providing absolute file-paths, sample IDs and read direction.

DATA PROCESSING: DADA2

The Diverse Amplicon Denoising Algorithm 2 (DADA2) plugin was used to provide sequence quality control and feature table construction (Callahan et al., 2016). Low quality sequence reads were removed through trimming and truncating processes, whereby the forward reads were trimmed 7 nucleotides from the left and truncated 250 nucleotides from the right. The reverse reads were timed 21 nucleotides from the left and truncated 250 nucleotides from the right. DADA2 also acts as a filter, to remove chimeric sequences that may act as a 'contaminant' when assessing sample biodiversity. Chimeric sequences are artefacts generated following incomplete amplicon extension during PCR amplification (Haas et al., 2011). The premature amplicon generated can then act as a primer in subsequent PCR cycles and form a chimeric sequence (Haas et al., 2011). Following further amplification of this sequence, a chimeric artefact will falsely represent novel organisms in the sample dataset (Haas et al., 2011). Consensus chimera filtering was applied to detect chimeric sequences in individual samples and remove those chimeras representing a sufficient fraction of that sample.

The non-chimeric amplicon sequence variants (ASV) table generated from the DADA2 pipeline displays the observation frequency of each ASV relative to individual samples. Use of ASV sequences has recently superseded previous construction of Operational Taxonomic Unit (OTUs) tables (Callahan et al., 2017). An ASV, previously referred to as an OTU, is a cluster of sequence reads that differ from each other by less than a fixed dissimilarity threshold, most commonly 3 % (Callahan et al., 2017). A higher resolution picture of ecological patterns within samples is thought to be demonstrated with ASV methods compared to OTU based methods. The *de novo*-based process of ASV inference employed by the DADA2 pipeline occurs prior to introduction of amplification and sequencing errors and is able to discriminate between sequence variants at even single nucleotide level (Callahan et al., 2017). The mafft pipeline from the q2-phylogeny plugin was used to perform multiple sequence alignment of the sequences in the resulting ASV feature table (Katoh & Standley, 2013). The alignment was

then masked to remove highly variable sequences and a midpoint rooted phylogenetic tree generated for use in further downstream analysis.

DATA ANALYSIS: ALPHA AND BETA DIVERSITY

The rooted phylogenetic tree generated was used to assess microbial community diversity both within and between samples. The QIIME2™ q2-diverity plugin was used to perform alpha and beta diversity analysis to a depth of 44, 841. The sampling depth used was determined based on the minimum frequency of sequences per sample, allowing for maximal retention of sequences per sample (Navas-Molina et al., 2013).

Alpha diversity analysis represents a commonly used tool for the composite measure of the number of species in relation to that species abundance within a given sample, with this being described as species richness and evenness. Alpha, or within-sample, diversity was analysed using the following core-metric parameters:

- *Pielou's Evenness index* – A measure of microbial community evenness. (Qiime2, 2019)
- *Observed ASV index* – A qualitative measure of community richness (Qiime2, 2019)
- *Shannons diversity index* – A quantitative measure of community richness using natural logarithm and accounting for both abundance and evenness of the taxa present. (Qiime2, 2019)

Statistical relevance of identified alpha diversity metrics and sample metadata groups was validated through pairwise ANOVA Kruskal-Wallis testing. Application of Kruskal-Wallis for this dataset was favourable since it makes no assumption of data distribution normality (non-parametric) and shows limited sensitivity regarding asymmetrical sample size (McCrum-Gardner, 2008). To account for multiple-testing, *p* - values were adjusted using the Benjamini-Hochberg correction, with these provided as *q* - values.

Often used in accordance with alpha diversity, beta diversity is a useful measure of taxonomic diversity between different samples. To compare patterns of beta diversity (diversity in bacterial community composition) among the different treatment groups, UniFrac (Unique

Fraction) distance matrices were calculated whereby greater values indicate greater sample dissimilarity (Anderson et al., 2011).

- *Unweighted UniFrac* – A qualitative measure of community dissimilarity considering only ASV presence or absence (community membership) (Qiime2, 2019).
- *Weighted UniFrac* – A quantitative measure of community dissimilarity considering ASV presence, absence and relative abundance (community structure) (Qiime2, 2019).

The incorporation of phylogenetic information within these UniFrac metrics allows for interpretation of the degree of divergence between sequences and improves model power (Lozupone & Knight, 2005). Utilising both weighted and unweighted UniFrac measures minimises the likelihood that individual taxonomic abundance would mask the identification of trends in community differences between the samples. Together, these UniFrac metrics are able to provide a comprehensive picture of sample taxonomic composition and microbial community shifts.

After UniFrac beta diversity statistics were determined, the Permutational Multivariate Analysis of Variance (PERMANOVA) was applied to each UniFrac metric to assess statistical significance against individual covariates of interest. No assumption of data normality is required in the application of PERMANOVA analysis, allowing this distance-based method to be widely used in the estimation of microbial community dissimilarity. Metrics were determined in comparison of different treatment groups. Microbial communities showing strong similarity had a Unifrac score tending toward 0 while microbial communities of higher divergence will have Unifrac scores tending toward 1.

The UniFrac dissimilarity statistics generated were presented using Principle Coordinates Analysis (PCoA), a distance-based plot assigning individual samples a specific location in three-dimensional space (Halko et al., 2011; legendre & legendre, 2012). PCoA plots are often used as a means of identifying overall similarity/dissimilarity between populations of samples according to covariates of interest. Samples with more similar population compositions will cluster closer together, while samples showing more compositional dissimilarity will be positioned farther apart in PCoA space. For determining differential abundance of specific taxa between sample populations or PCoA clusters, further abundance testing was performed.

DATA ANALYSIS: TAXONOMIC COMPOSITION

ASVs were assigned taxonomy using the QIIME2™ q2-feature-classifier plugin with the pre-trained Naïve Bayes Greengenes 13_8 99% classifier (16S rRNA) (Pedregosa et al., 2011). A QIIME2™ taxa bar-plot was generated to view the assigned taxonomic composition of individual samples according to sample metadata variates of interest. A venn diagram was created using MetaCoMETs jvenn programme (Bardou et al., 2014) to compare the microbiome data from each treatment group. The core microbiome can be represented within the shared overlapping regions.

Linear Discriminant Analysis (LDA) effect size (LEfSe) methods were used to identify differentially abundant features across sample groups (Segata et al., 2011). LEfSe utilises the two-tailed nonparametric Kruskal-Wallis test to first evaluate the differential between treatment groups, with all features not agreeing with the null hypothesis undergoing pairwise Wilcoxon testing. Finally, an LDA model was constructed, from which the relative difference of this feature among different groups was used as a rank. Microbial features were considered significant if they had a $p < 0.05$ and an LDA score ($10g_{10}$) > 3.

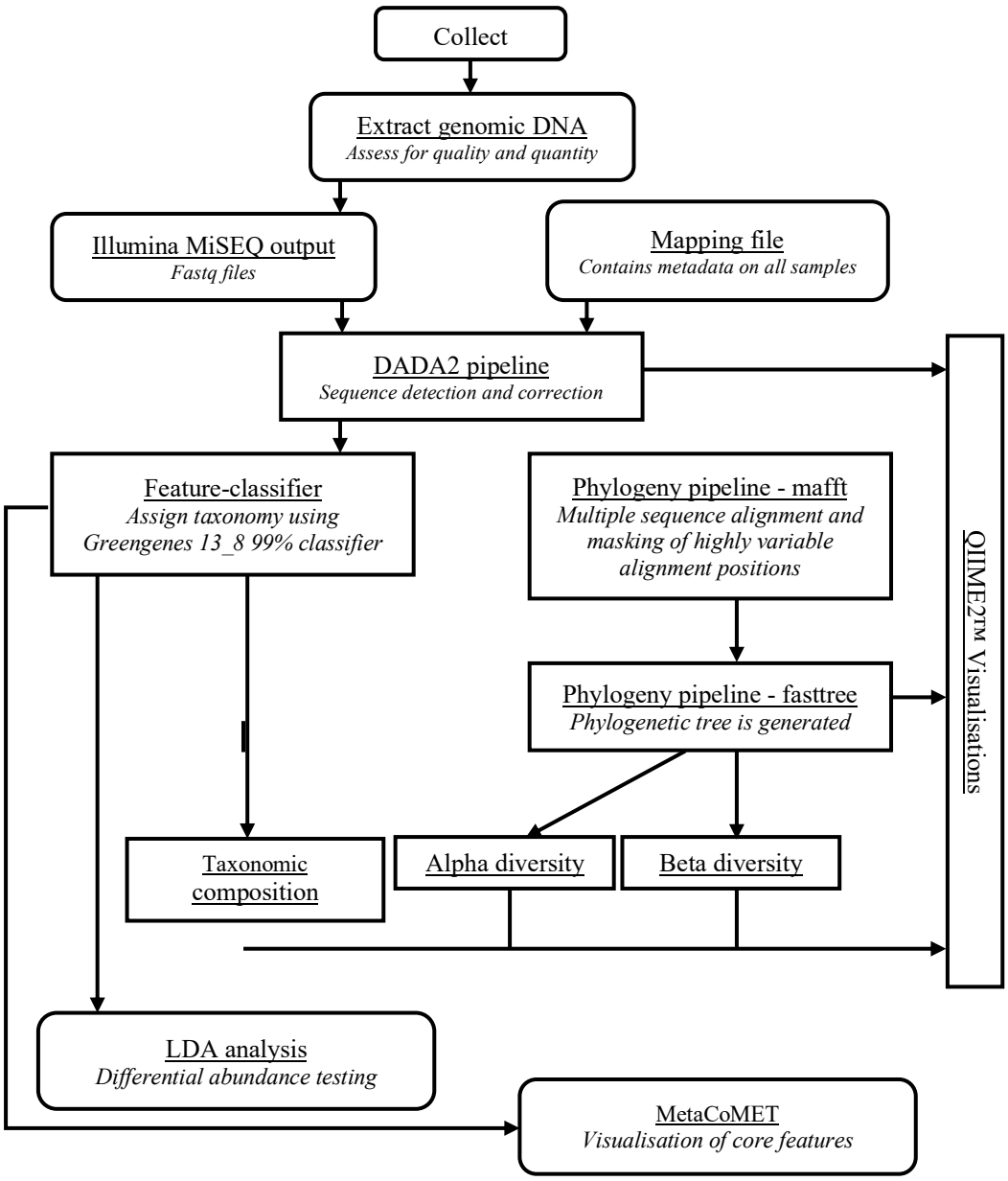


Figure 66. Analysis workflow showing sample collection and data processing pathways

RESULTS

DNA SAMPLES

DNA was extracted from all samples collected using protocols previously described, resulting in a submission sample size of 56 samples for 16S rRNA Illumina MiSeq processing. All samples were checked for DNA isolation and integrity using agarose gel electrophoresis before being quantified using Qubit dye-based methodologies. Results from DNA quantification and are provided in Appendix 5.

MICROBIOTA DIVERSITY ANALYSIS

The 56 samples processed via 16S rRNA techniques gave an average of 287,108 raw data reads per sample. Downstream application of denoising and chimera removal QIIME2™ pipelines refined these raw data reads, deriving a total of 4,893,551 sequences across all samples, with an average of 87,385 sequences per sample. Sequence frequency per sample had a range of 79,148 sequences with minimum and maximum counts per sample being 44,841 and 123,989 respectively. Detail on specific sequence counts attained per sample is provided in Appendix XX. The 4,893,551 sequences presented 724 differential features or ASVs. Of the 724 ASV clusters, 11 (2 %) were assigned to Kingdom, 29 (4 %) Order, 110 (15 %) Family, 41 (6 %) Genus and 533 (73 %) to Species.

ALPHA DIVERSITY ANALYSIS

Alpha rarefaction was used to ascertain the correct sampling depth for downstream analysis, by visualisation of the number of samples that are retained within the analysable dataset at varying rarefaction sampling depths (Figure 67). A sampling depth of 44,841 was used, retaining all samples and 51.31% (2,511,096) of the total sequences in the analysis when sampling depth was required.

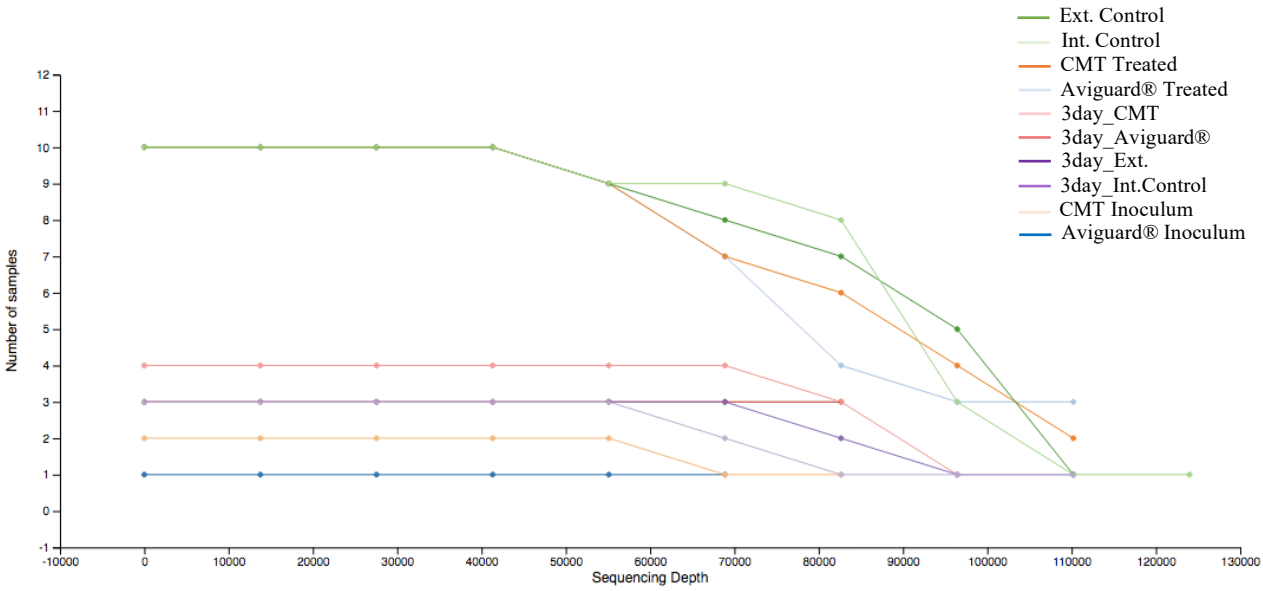


Figure 67. The number of individual samples retained per treatment group following alpha rarefaction at specified sequencing depths. At 3 d.p.h (Aviguard n=3; Ext. control n=3; Int. control n=3; CMT n=4) and 7 d.p.h (7day) (all treatment groups n=10)

Observed ASV metric

Observed ASV alpha diversity metrics were assessed as an initial primary assessment of individual sample microbial community richness (Figure 68a). Analysis was first directed at identifying significant differences in the number of unique observed ASVs between the 3 d.p.h microbiota from each treatment group, with these groups being defined from this point forward as; 3 d.p.h CMT, 3 d.p.h Aviguard®, 3 d.p.h Internal Control and 3 d.p.h External Control. Unique ASV frequencies derived for each 3 d.p.h treatment group ranged from 29 – 139 ASVs, with Internal (29 ASVs) and External (34 ASVs) control groups showing the lowest ASV frequencies and the CMT treated group (139 ASVs) showing the greatest. Microbiota from Aviguard® treated birds returned a total of 58 unique sequence variants. Treatment group did not significantly alter observed ASV using pairwise Kruskal-Wallis analysis (corrected for FDR) ($q > 0.05$), likely due to the small sample size used.

The second focus was towards microbiota samples from 7 d.p.h birds of each treatment group. From this point forward defined as; 7 d.p.h CMT, 7 d.p.h Aviguard®, 7 d.p.h Internal Control, 7 d.p.h External Control. Alpha diversity analysis of observed ASVs showed an ASV count ranging from 83 – 328 per treatment group. As with 3 d.p.h samples, lowest unique ASV frequencies were found in Internal control (119 ASVs) and External control (83 ASVs)

treatment group samples, with 7 d.p.h Aviguard® (144 ASVs) and 7 d.p.h CMT (328 ASVs) microbiota having substantially more. Pairwise Kruskal-Wallis testing between each of the 7 d.p.h treatment groups showed 7 d.p.h Aviguard® microbiota had significantly more observed ASVs compared to 7 d.p.h Internal control (H = 9.15; q = 0.02) and 7 d.p.h Ext. control (H = 14.31; q = 0.00) microbiota (
Table 26). Similarly, 7 d.p.h CMT microbiota was significantly distinct according to this diversity metric to both 7 d.p.h Int. control (H = 14.30; q = 0.00) and 7 d.p.h Ext. control (H = 14.31; q = 0.00) microbiota. Pairwise comparison of 7 d.p.h Aviguard® and 7 d.p.h CMT sample groups identified CMT samples as having significantly higher frequency of observed ASVs (H = 14.28; q = 0.00). Individual ASV counts per sample are provided in Appendix 5.

Inoculum material given to Aviguard® and CMT treatment groups were assessed for sample microbial composition and diversity and from this point forward will be defined as CMT Inoculum and Aviguard® Inoculum. Alpha diversity parameters were not applied to the same depth as for 3 d.p.h and 7 d.p.h treatment group samples due to small inoculum sample populations. The observed ASV alpha diversity metric was applied to inoculum samples and returned 392 observed ASVs for CMT inoculum and 92 ASVs for Aviguard® inoculum, although this variation was not statistically significant by pairwise significance testing (H = 1.25; q = 0.24).

Shannon's diversity metric

Shannon alpha diversity metrics were calculated to derive a more comprehensive understanding of the abundance of each of the Observed ASV's within individual samples. Shannon values ranged from 1.44 – 4.33 for 3 d.p.h treatment group samples and 1.62 – 5.78 for 7 d.p.h treatment group samples (Appendix 5). After FDR adjustment of p - values, treatment group resulted in no significant divergence through pairwise comparison of 3 d.p.h data (q > 0.05) (Figure 68b) (
Table 26).

Shannon diversity was significantly higher in 7 d.p.h Aviguard® treatment samples compared to that of the 7 d.p.h Internal control (H = 10.08; q = 0.01) and 7 d.p.h External control (H = 8.69; q =0.01) groups (Table 2). At 7 d.p.h, the CMT microbiome had higher average Shannon values compared to 7 d.p.h Int. control (H = 14.29; q = 0.00) and Ext. control (H = 14.29; q =

0.00) groups. CMT treatment did not significantly alter Shannon diversity of samples compared to that of Aviguard® samples at 7 d.p.h (H = 7.00; q = 0.05).

Pielou's evenness metric

Pielou's Eveness metric works to incorporate both differential ASV number with individual ASV abundance to create a metric of the evenness of ASV distribution within a sample. Measured on a scale of 0 to 1, the closer an evenness value is to 1, the more evenly spread abundancy is within that sample. Derived evenness values ranged from 0.36 - 0.70 for 3 d.p.h treatment groups, with no statistically significant difference in group evenness following pairwise Kruskal-Wallis analysis of all 3 d.p.h treatment groups (q > 0.05) (Figure 68c) (
Table 26). Treatment groups of 7 d.p.h microbiota had Pielou evenness scores ranging from 0.38 – 0.77, showing little variation in microbiota community evenness between different treatment groups. 7 d.p.h CMT treated birds showed significantly more evenly distributed microbial communities compared to Internal control (H = 10.08; q = 0.03) and External control (H = 10.08 q = 0.03) populations, with evenness values per sample provided in Appendix 5.

Table 26. Summary statistics of alpha diversity analysis according to sample treatment group. Pairwise comparisons of alpha diversity metrics were calculated through ANOVA Kruskal-wallis testing. All p-values were adjusted using Benjamini-Hochberg correction, with these provided as q-values.

Cull	Group comparisons		Pairwise kruskal-wallis					
			Observed ASV		Shannon		Pielou's Evenness	
	Group 1	Group 2	*H*-value	*q*-value	*H*-value	*q*-value	*H*-value	*q*-value
3 d.p.i	Aviguard®	CMT	1.13	0.31	0.00	1.00	1.13	0.37
		Ext. Control	3.86	0.09	3.86	0.09	3.86	0.14
		Int. Control	3.86	0.09	3.86	0.09	3.86	0.14
	CMT	Ext. Control	4.50	0.07	4.50	0.07	4.50	0.12
		Int. Control	4.50	0.07	4.50	0.07	0.50	0.58
	Ext. Control	Int. Control	3.86	0.09	0.05	0.09	2.33	0.21
7 d.p.i	Aviguard®	CMT	14.28	0.00	7.00	0.05	0.14	0.74
		Ext. Control	14.31	0.00	8.69	0.04	7.00	0.06
		Int. Control	9.15	0.02	10.08	0.02	7.00	0.06
	CMT	Ext. Control	14.31	0.00	14.29	0.00	10.57	0.03
		Int. Control	14.30	0.00	14.29	0.00	10.08	0.03
	Ext. Control	Int. Control	4.66	0.07	5.49	0.06	0.14	0.74
Inoculum	CMT Inoculum	Aviguard® Inoculum	1.50	0.24	N/A		N/A	

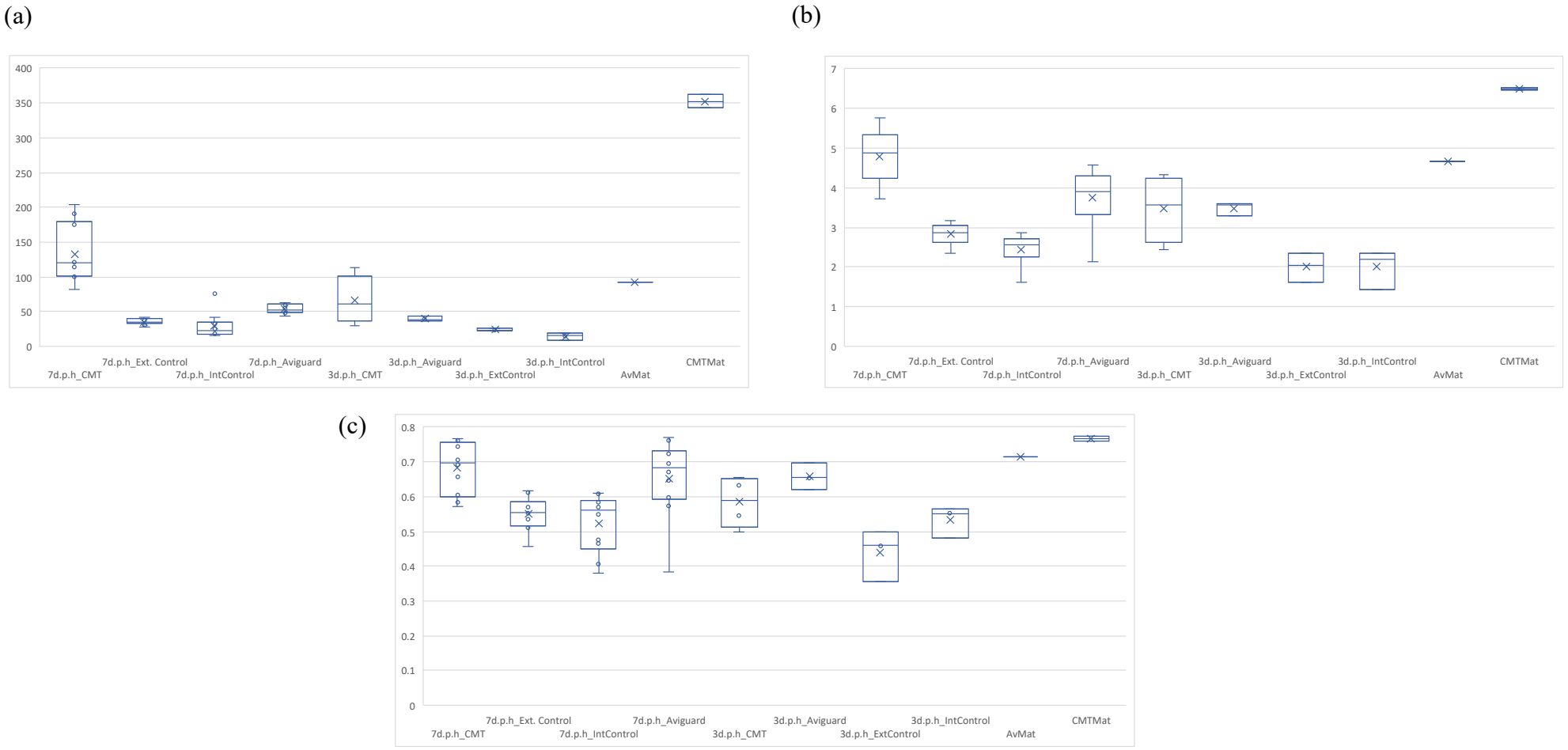


Figure 68. Box and whisker plot of alpha diversity metrics showing (a) observed ASV metric (b) shannon diversity metric (c) pielou's evenness metric according to specific treatment group. 3 d.p.h (Aviguard n=3; Ext. control n=3; Int. control n=3; CMT n=4) and 7 d.p.h (7day) (all treatment groups n=10)

BETA DIVERSITY ANALYSIS

Beta diversity metrics were calculated with the purpose of determining differences and similarities between the microbiota community membership and the community structure of different treatment groups. Analysis of both weighted and unweighted Unifrac measurements was essential to appropriately explore microbial diversity, with statistical significance of pairwise comparisons provided in Table 27.

Unweighted Unifrac beta diversity

Pairwise PERMANOVA comparisons of taxa presence or absence within 3 d.p.h treatment groups (unweighted Unifrac) revealed no significant divergence between any treatment group ($q > 0.05$), with significance values for each comparison provided in Table 27. Performing such pairwise comparisons on 7 d.p.h microbiota samples shows dissimilarity of statistical significance ($q = 0.01$) when comparing both the 7 d.p.h Aviguard® and 7 d.p.h CMT microbiome with 7 d.p.h Internal and External control microbiota samples. Pairwise PERMANOVA comparison of Aviguard® and CMT Inoculum material yielded no level of significant dissimilarity between the two microbial communities (*Pseudo-F* = 61.90; $q = 0.34$).

Using a PCoA transformation of unweighted Unifrac matrices showed distinct spatial clustering of both Aviguard® treated and External control samples at 3 d.p.h compared to the higher dispersal seen of 3 d.p.h CMT and Internal control samples. By 7 d.p.h it is External control and CMT treated samples that show visibly greater spatial clustering. CMT inoculum appears to form stronger clustering with samples from CMT treated birds than the relationship seen for Aviguard® inoculum and samples from Aviguard® treated birds (Figure 69).

Weighted Unifrac beta diversity

Relative ASV abundance was incorporated to weight the UniFrac measurement (weighted UniFrac) and minimise the influence of low abundance ASV's. Pairwise PERMANOVA comparisons of weighted Unifrac metrics for 3 d.p.h treatment group samples revealed no significant effect of specific treatment on sample beta diversity ($q > 0.05$), with significance values for each comparison provided in Table 27.

Pairwise comparisons of 7 d.p.h treatment groups showed both CMT and Aviguard® treated birds had significantly distinct caecal microbial communities compared to both Internal and External control groups (q = 0.01). Comparison of microbiota from 7 d.p.h CMT and 7 d.p.h Aviguard® treated birds showed significant effects of treatment on beta diversity using this metric (*Pseudo-F* = 9.72; q = 0.01). No such impact was observed between the beta diversity of 7 d.p.h Internal and 7 d.p.h External control groups when directly compared (*Pseudo-F* = 2.37; q = 0.14).

Pairwise PERMANOVA comparison of Aviguard® and CMT Inoculum material yielded no level of significant dissimilarity between the two microbial communities via weighted UniFrac analysis (*Pseudo-F* = 794.23; q = 0.35). All UniFrac distances were plotted using PCoA transformation. While no obvious clustering could be identified for 3 d.p.h samples, both 7 d.p.h internal and external control groups appear to show less dispersal compared to CMT and Aviguard® samples. As with unweighted Unifrac measures, CMT inoculum shows stronger clustering with samples from CMT treated birds compared to Aviguard® inoculum with samples from Aviguard® treated birds (Figure 70).

Table 27. Summary statistics of beta diversity analysis according to sample treatment group. *P*-values show pairwise PERMANOVA comparisons of Unweighted and Weighted Unifrac measures. All *p*-values were adjusted using Benjamini-Hochberg correction, with these provided as *q*-values.

Cull	Group comparisons		PERMANOVA			
			Unweighted Unifrac		Weighted Unifrac	
	Group 1	Group 2	*Pseudo-F*	*q*-value	*Pseudo-F*	*q*-value
3 d.p.h	Aviguard®	CMT	3.04	0.07	1.67	0.23
		Ext. Control	7.83	0.12	8.77	0.14
		Int. Control	2.46	0.12	6.41	0.13
	CMT	Ext. Control	3.21	0.11	6.15	0.09
		Int. Control	1.77	0.1	5.19	0.1
	Ext. Control	Int. Control	1.95	0.12	0.93	0.43
7 d.p.h	Aviguard®	CMT	11.99	0.01	9.72	0.01
		Ext. Control	9.26	0.01	15.72	0.01
		Int. Control	4.22	0.01	14.71	0.01
	CMT	Ext. Control	24.69	0.01	36.70	0.01
		Int. Control	13.07	0.01	34.58	0.01
	Ext. Control	Int. Control	5.48	0.01	2.37	0.14
Inoculum	CMT Inoculum	Aviguard® Inoculum	61.90	0.34	794.23	0.35

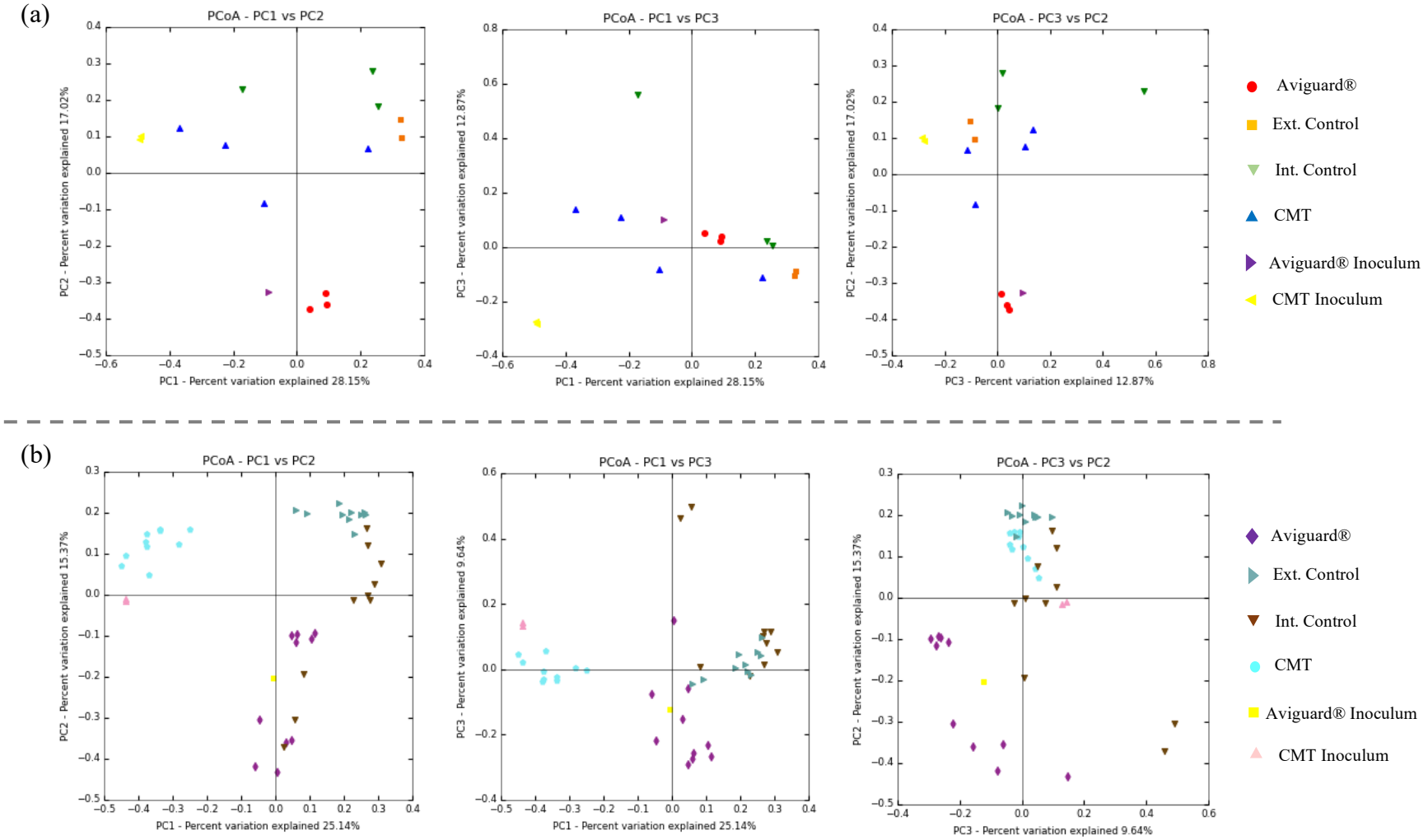


Figure 69. Principal coordinate analysis (PCoA) plots based on Unweighted Unifrac distances for all samples collected at 3 d.p.h (Aviguard n=3; Ext. control n=3; Int. control n=3; CMT n=4) and 7 d.p.h (7day) (all treatment groups n=10) alongside Aviguard® (AviguardInoc; n=1) and CMT inoculum (CMT Inoc; n=2) material.

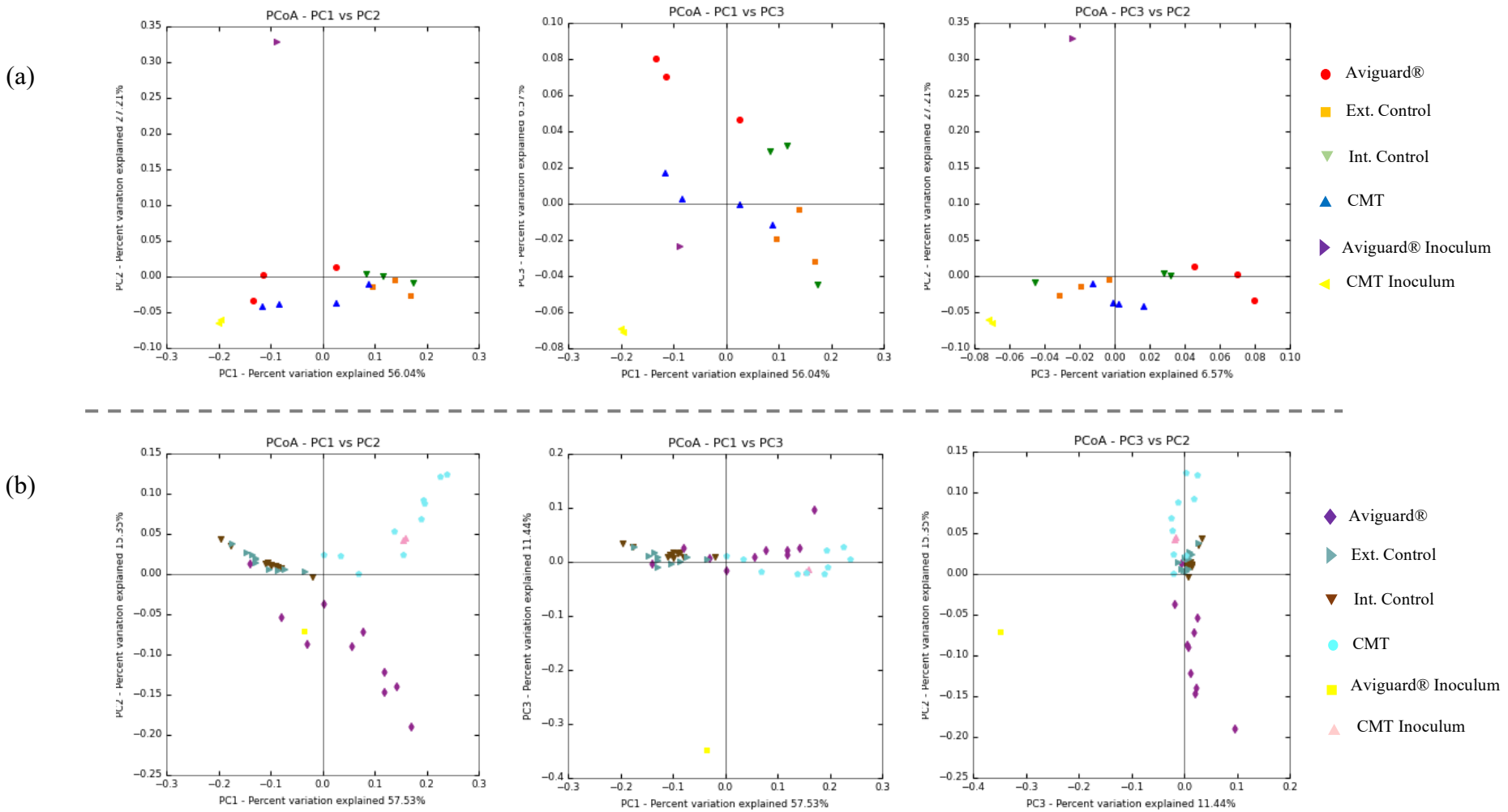


Figure 70. Principal coordinate analysis (PCoA) plots based on Weighted Unifrac distances for all samples collected at 3 d.p.h (Aviguard n=3; Ext. control n=3; Int. control n=3; CMT n=4) and 7 d.p.h (7day) (all treatment groups n=10) alongside Aviguard® (AviguardInoc; n=1) and CMT inoculum (CMT Inoc; n=2) material.

CAECAL COMMUNITY TAXONOMIC COMPOSITION

Taxonomic classification applied to all samples revealed a small group of identified sequences that could not be classified to any Kingdom, making up 0.0003% of the total relative abundance. A further sequence group were classified to the taxonomic kingdom level Bacteria but were unable to be further classified, with this bacterial group comprising 0.0012% of the total relative abundance. Classification of assigned taxonomies at Class level identified 25 bacterial Classes, with each being present in at least one sample. Accounting for all samples, the relative abundance of two taxonomic classes predominated, with these being *Clostridia* (52.27 %) and *Gammaproteobacteria* (42.10 %).

Further taxonomic classification to family level identified 68 different family taxons. An additional two groups were unable to be assigned taxonomic classification further than Order, with these comprising 0.01 % and 1.36 % of the overall relative abundance taking in to account all samples. Three taxonomic families comprised the majority of the total relative abundance for all samples, being *Enterobacteriaceae* (42.10 %), *Lachnospiraceae* (28.20 %) and *Ruminococcaceae* (16.13 %). Figures detailing relative abundance of taxonomic classifications for each sample are provided at Class level in Figure 71 and Family level in Figure 72.

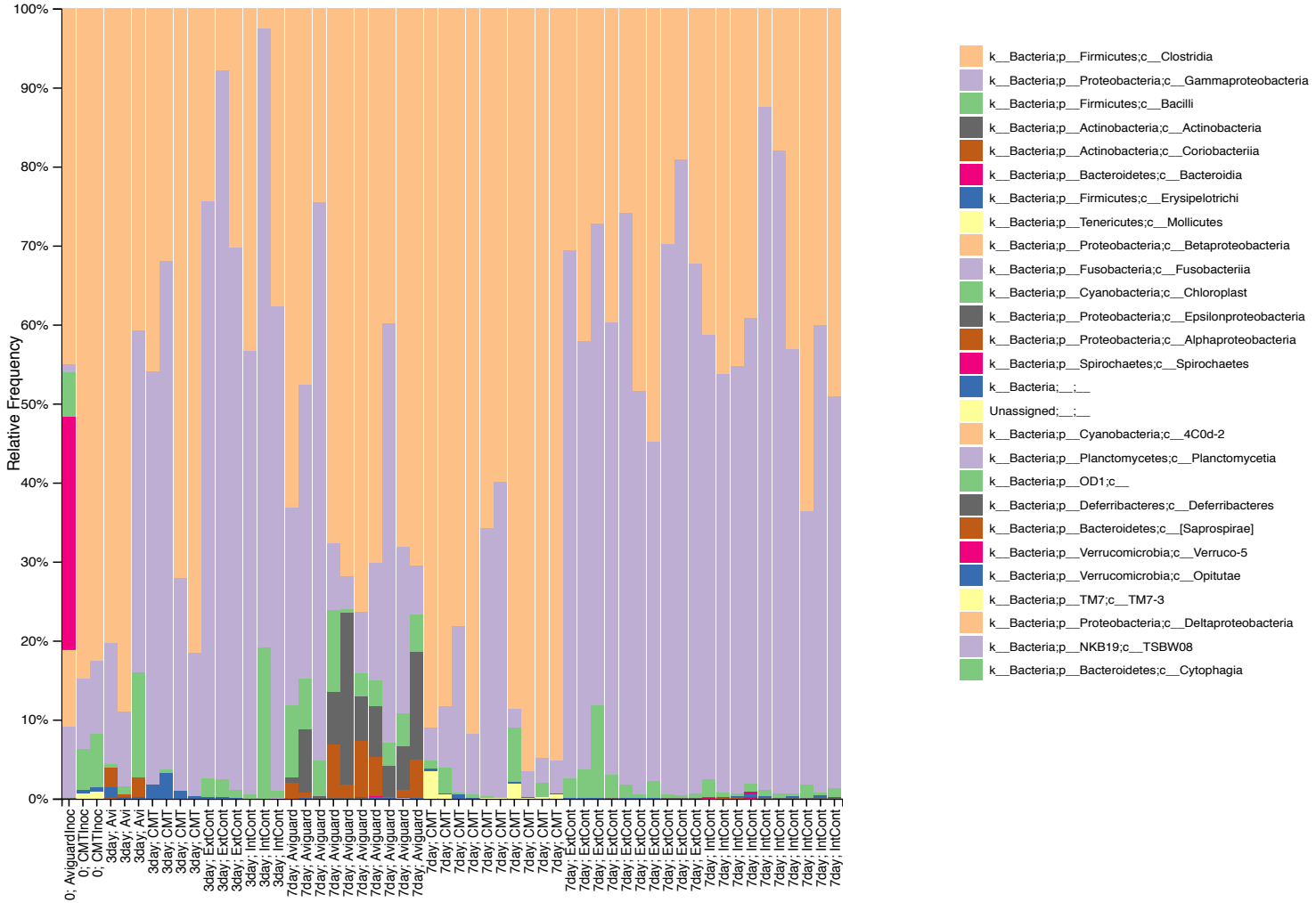


Figure 71. Taxa plot showing the relative abundance at bacterial Class taxonomic level within the chicken caecal microbiota. Samples are given according to treatment group as 3 d.p.h (3day) (Aviguard n=3; Ext. control n=3; Int. control n=3; CMT n=4) and 7 d.p.h (7day) (all treatment groups n=10) alongside Aviguard® (AviguardInoc; n=1) and CMT inoculum (CMT Inoc; n=2) material.

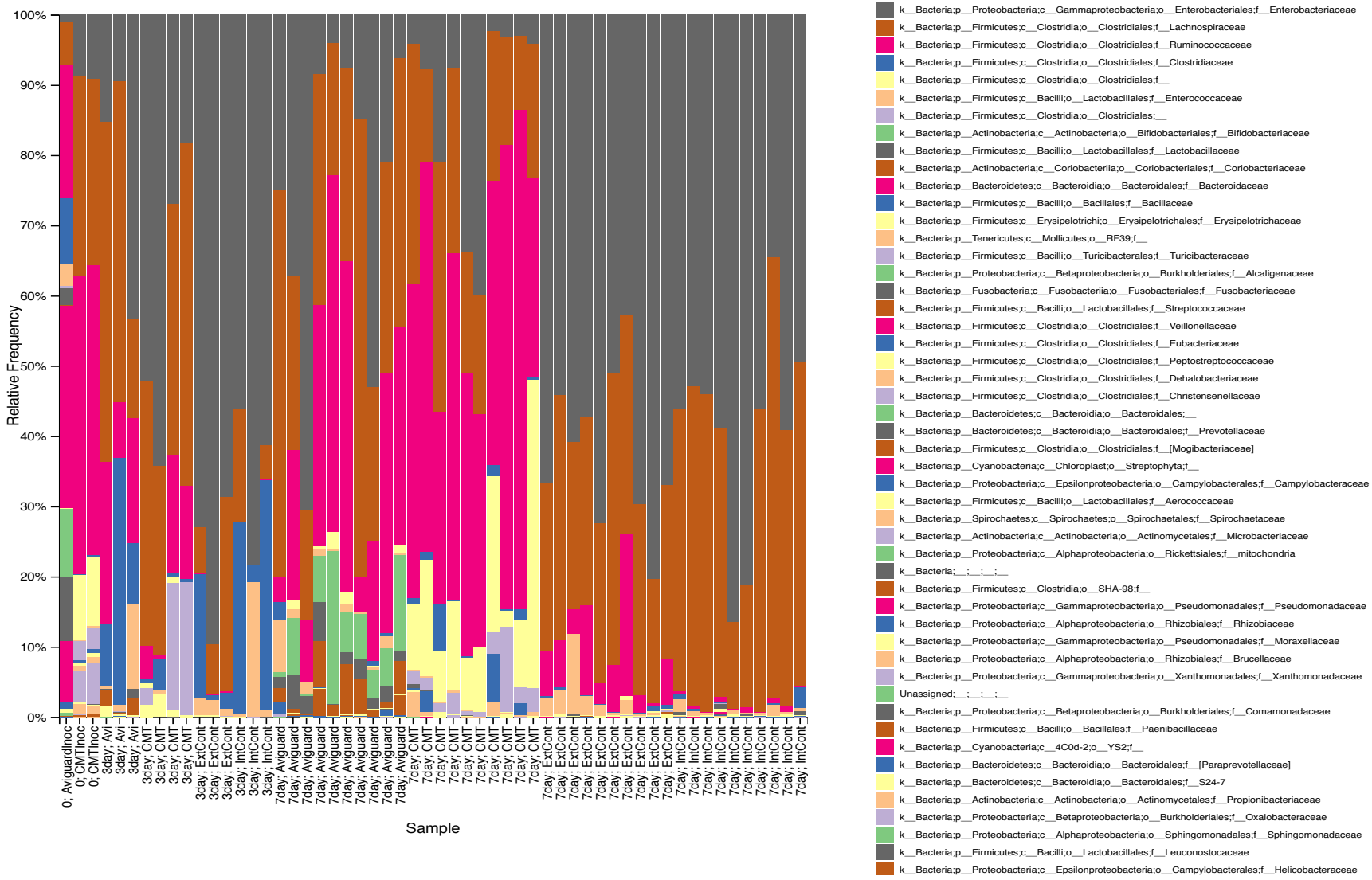


Figure 72. Taxa plot showing the relative abundance at bacterial Family taxonomic level within the chicken caecal microbiota. Samples are given according to treatment group as 3 d.p.h (Aviguard n=3; Ext. control n=3; Int. control n=3; CMT n=4) and 7 d.p.h (7day) (all treatment groups n=10) alongside Aviguard® (AviguardInoc; n=1) and CMT inoculum (CMT Inoc; n=2) material.

Taxonomic level: Class

Considering only taxa with > 2 % relative abundance averaged over treatment group, 3 d.p.h microbial communities belonged to 3 taxonomic classes and 7 d.p.h communities to 5. Looking first at samples from 3 d.p.h treatment groups, *Clostridia* and *Gammaproteobacteria* predominated, with an average relative abundance of 44.18 % and 51.32 % across all 3 d.p.h samples, respectively. *Clostridia* was the predominant taxonomic class in both 3 d.p.h Aviguard® (70.02 ± 25.73 %) and 3 d.p.h CMT (57.90 ± 22.92 %), treatment groups, while *Gammaproteobacteria* predominated in both 3 d.p.h Internal (65.20 ± 11.64 %) and 3 d.p.h External (77.07 ± 11.11 %) control microbiota. Similarly for 7 d.p.h samples, Aviguard® and CMT treated birds showed microbiota with communities rich in *Clostridia* (60.03 ± 16.92 %; 85.03 ± 12.87 %, respectively). 7 d.p.h Internal and External control microbiota was instead more dominantly composed of *Gammaproteobacteria* (58.91 ± 14.95 %; 62.21 ± 11.18 % respectively.

Clostridia taxons represented a large proportion of the CMT inoculum samples (83.73 ± 1.67 %) with *Gammaproteobacteria* comprising 9 ± 0.25 % and *Bacilli* 6.05 ± 1.17 %. Aviguard® inoculum showed a more diverse composition, being largely composed of five different taxonomic classes; *Clostridia* (45.06 %), *Bacteroidia* (29.42 %), *Betaproteobacteria* (9.69 %), *Fusobacteriia* (9.12 %) and *Bacilli* (5.56 %). Three bacterial taxonomic Classes (*Bacteroidia, Fusobacteriia and Betaproteobacteria*) collectively comprised 48.20 % of the Aviguard® inoculum material, however showed 0.00 % relative abundance within the microbiota samples collected from Aviguard® treated birds at 7 d.p.h. Representation of each taxonomic class (with relative abundance of > 2% in at least one treatment group) within treatment groups at 3 d.p.h and 7 d.p.h are provided in Table 28 accompanied by visible representation in Figure 75.

Taxonomic level: Family

Further in-depth assessment of family taxonomic classifications, again considering only taxa with > 2 % relative abundance averaged over treatment group, showed 3 d.p.h microbial communities belonged to 6 family taxons, while 7 d.p.h communities belonged to 9. Of the *Gammaproteobacteria* that dominated Internal and External control samples at Class taxonomic level, classification to Family taxonomic level found this to be almost exclusively bacterial families of *Enterobacteriaceae*. Of the clostridia that were highly represented in all

samples, but particularly dominant in Aviguard® and CMT treated birds, Family level classification divided this class into *Lachnospiraceae* and *Ruminococcaceae* families.

Microbiota of 3 d.p.h samples comprised primarily of three Family taxons; being *Enterobacteriaceae* (51.32 ± 21.20 %), *Lachnospiraceae* (23.61 ± 15.43 %) and *Clostridiaceae* (11.72 ± 9.03 %). *Enterobacteriaceae* constituted a larger proportion of 3 d.p.h control group samples, being 65.19 (± 11.62) % of Internal control communities and 77.07 (± 11.11) % of External control communities. The relative abundance of *Enterobacteriaceae* was considerably lower in microbial communities of 3 d.p.h CMT treated samples (40.37 ± 21.49 %) and 3 d.p.h Aviguard® treated samples (22.65 ± 18.07 %). The opposite was true for both *Lachnospiraceae* and *Clostridiaceae* taxons. *Lachnospiraceae* comprised 36.12 (± 18.98) % of 3 d.p.h Aviguard®, 37.39 (± 9.01) % of 3 d.p.h CMT, 7.06 ± 8.28 % of 3 d.p.h Internal control and 13.89 (± 12.07) % of 3 d.p.h External control microbiota while *Clostridiaceae* comprised 17.62 ± 15.27 % of 3 d.p.h Aviguard®, 1.53 ± 1.92 % of 3 d.p.h CMT, 20.8 (± 16.04) % of 3 d.p.h Internal control and 6.89 (± 9.43) % of 3 d.p.h External control microbiota. *Ruminococcaceae* taxons were relatively highly represented in both 3 d.p.h Aviguard® (16.22 ± 7.65 %) and 3 d.p.h CMT (8.79 ± 7.48 %) microbial communities, whilst showing little contribution to both 3 d.p.h Internal control (0.03 ± 0.04 %) and 3 d.p.h External control (0.06 ± 0.06) microbial communities.

Enterobacteriaceae and *Lachnospiraceae* were the predominant families in 7 d.p.h microbial communities sampled, comprising an average relative abundance of 39.65 ± 24.68 % and 30.14 ± 7.70 % across all 7 d.p.h treatment groups respectively. *Ruminococcaceae* taxons contributed an average of 19.65 ± 20.30 % across all 7 d.p.h treatment groups. Comparing 7 d.p.h microbial community composition according to specific treatment group, *Lachnospiraceae* showed relatively even representation across treatment groups (ranging from 21.00 ± 8.50 % - 39.03 ± 14.50 %). *Clostridia Ruminococcaceae* showed higher relative abundance in both 7 d.p.h Aviguard® (25.54 ± 17.03 %) and 7 d.p.h CMT (45.59 ± 15.03 %) compared to 7 d.p.h Internal control (0.38 ± 0.33 %) and External control (7.11 ± 6.55 %) groups. An inverse relationship was observed for *Enterobacteriaceae* within 7 d.p.h microbial communities, with Internal control (58.91 ± 14.95 %) and External control (62.21 ± 11.18 %) microbiota having higher relative abundance when compared to microbial communities from Aviguard® (24.78 ± 22.38 %) and CMT (12.71 ± 13.94 %) treated birds.

Aviguard® inoculum was comprised of 9 taxonomic families with a treatment group averaged relative abundance of > 2 %, with CMT inoculum represented by 6. While *Lachnospiraceae* was fairly evenly represented in the microbiota of different treatment groups, it was considerably lower in relative abundance within Aviguard® inoculum compared to CMT inoculum (6.16 and 27.29 ± 1.31 % respectively). Although present in both inoculum material, *Ruminococcaceae* was considerably more represented in CMT inoculum (41.85 ± 0.82 %) compared to that of Aviguard® inoculum (19.05 %). Family taxon representation averaged by treatment group (for taxons with group representation > 2 %) is provided in Table 29 and a visible representation in Figure 74.

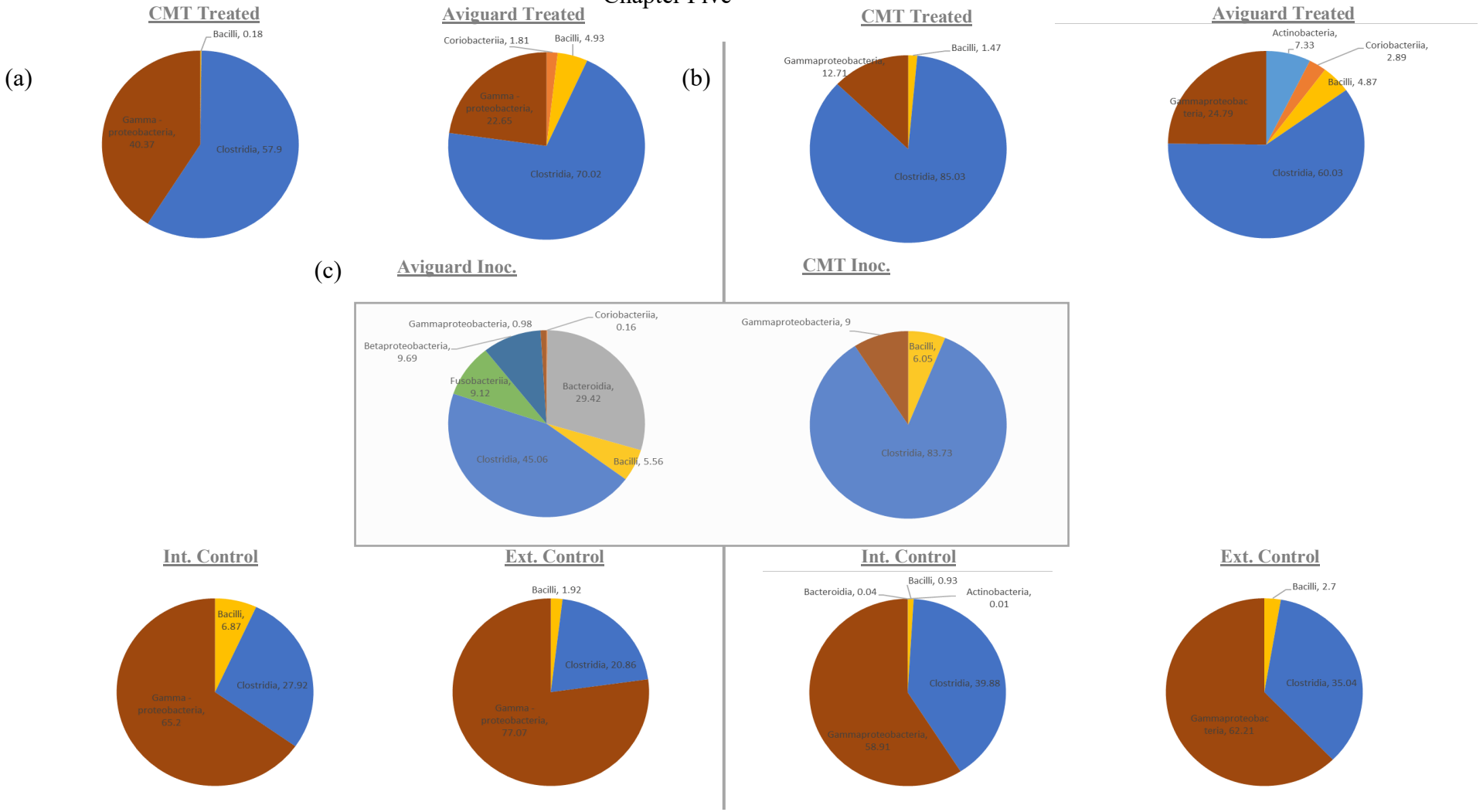


Figure 73. Average relative abundance of assigned bacterial Classes according to treatment group at (a) 3 d.p.h (Aviguard n=3; Ext. control n=3; Int. control n=3; CMT n=4) and (b) 7 d.p.h (all treatment groups n=10) alongside (c) Aviguard® (AviguardInoc; n=1) and CMT inoculum (CMT Inoc; n=2) material. Only Classes with > 2 % relative abundance in at least one treatment group are shown.

Table 28. Relative abundance with associated SD of bacterial Classes within specific treatment groups of caecal or inoculate material collected at 3 d.p.h and 7 d.p.h. Only classes with > 2 % relative abundance in at least one treatment group are shown.

				Relative abundance (%) ± SD									
				CMT		Aviguard®		Int. Control		Ext. Control		Inoculum	
	Kingdom	Phylum	Class	3d.p.h	7d.p.h	3d.p.h	7d.p.h	3d.p.h	7d.p.h	3d.p.h	7d.p.h	Aviguard®	CMT
Relative abundance of taxa per treatment group (%) ± SD	***Bacteria***	***Actinobacter***	***Actinobacteria***	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	7.33 ± 6.35	0.00 ± 0.00	0.01 ± 0.01	0.00 ±0.00	0.00 ± 0.00	0.00	0.00 ± 0.00
	Bacteria	***Actinobacteria***	***Coriobacteriia***	0.00 ± 0.00	0.00 ± 0.00	1.81 ± 1.30	2.89 ± 2.77	0.00 ± 0.00	0.00 ±0.00	0.00 ± 0.00	0.00 ± 0.00	0.16	0.00 ± 0.00
	Bacteria	***Bacteroidetes***	***Bacteroidia***	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	29.42	0.00 ± 0.00
	Bacteria	***Firmicutes***	***Bacilli***	0.18 ±0.22	1.47 ± 2.20	4.93 ± 7.28	4.87 ± 2.99	6.87 ± 10.60	0.93 ± 0.62	1.92 ± 0.72	2.70 ± 3.39	5.56	6.05 ± 1.17
	Bacteria	***Firmicutes***	***Clostridia***	57.90 ± 22.92	85.03 ± 12.87	70.02 ± 25.73	60.03 ± 16.92	27.92 ± 22.12	39.88 ± 14.77	20.86 ± 11.58	35.04 ±11.04	45.06	83.73 ± 1.67
	Bacteria	***Fusobacteria***	***Fusobacteriia***	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	9.12	0.00 ± 0.00
	Bacteria	***Proteobacteria***	***Betaproteobacteria***	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ±0.00	0.00 ± 0.00	9.69	0.00 ± 0.00
	Bacteria	***Proteobacteria***	***Gamma proteobacteria***	40.37 ± 21.49	12.71 ± 13.94	22.65 ± 18.07	24.79 ± 22.35	65.20 ± 11.64	58.91 ± 14.95	77.07 ± 11.11	62.21 ± 11.18	0.98	9.00 ± 0.25

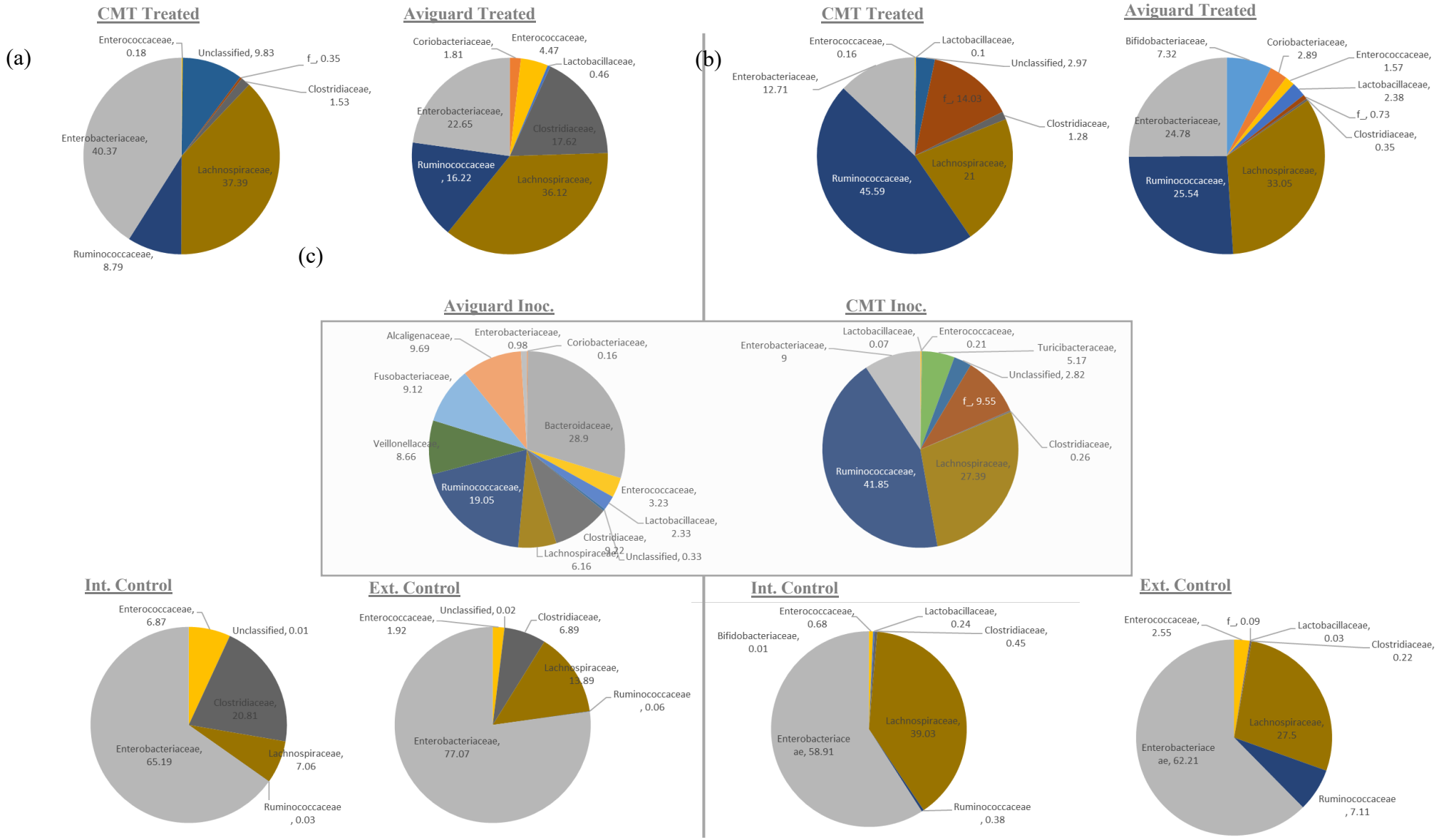


Figure 74. Average relative abundance of assigned bacterial Families according to treatment group at (a) 3 d.p.h (Aviguard n=3; Ext. control n=3; Int. control n=3; CMT n=4) and (b) 7 d.p.h (all treatment groups n=10) alongside (c) Aviguard® (AviguardInoc; n=1) and CMT inoculum (CMT Inoc; n=2) material. Only Classes with > 2 % relative abundance in at least one treatment group are shown.

Table 29. Relative abundance with associated SD of bacterial Families within specific treatment groups of caecal or inoculate material collected at 3 d.p.h and 7 d.p.h. Only classes with > 2 % relative abundance in at least one treatment group are shown.

	Taxonomic group			Relative abundance (%) ± SD									
				CMT		Aviguard®		Int. Control		Ext. Control		Inoculum	
	Class	Order	Family	3d.p.h	7d.p.h	3d.p.h	7d.p.h	3d.p.h	7d.p.h	3d.p.h	7d.p.h	Aviguard®	CMT
Relative abundance of taxa per treatment group (%) ± SD	*Actinobacteiia*	*Bifidobacteriales*	*Bifidobacteriaceae*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	7.32 ± 6.35	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00	0.00 ± 0.00
	Coriobacteriia	*Coriobacteriales*	*Coriobacteriaceae*	1.81 ± 1.30	0.00 ± 0.00	0.00 ± 0.00	2.89 ± 2.77	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.16	0.00 ± 0.00
	Bacteroidia	*Bacteroidales*	*Bacteroidaceae*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	28.90	0.00 ± 0.00
	Bacilli	*Lactobacillales*	*Enterococcaceae*	4.47 ± 6.63	0.16 ± 0.14	0.18 ± 0.22	1.57 ± 2.17	6.87 ± 10.60	0.68 ± 0.58	1.92 ± 0.72	2.55 ± 3.36	3.23	0.21 ± 0.03
	Bacilli	*Lactobacillales*	*Lactobacillaceae*	0.46 ± 0.66	0.10 ± 0.21	0.00 ± 0.00	2.38 ± 1.65	0.00 ± 0.00	0.24 ± 0.30	0.00 ± 0.00	0.03 ± 0.08	2.33	0.07 ± 0.01
	Bacilli	*Turicibacteraceae*	*Turicibacteraceae*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.06	0.00	5.10 ± 1.01
	Clostridia	*Clostridiales*	*Unclassified*	0.00 ± 0.00	2.97 ± 3.40	9.83 ± 10.06	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.02 ± 0.02	0.00 ± 0.00	0.33	2.82 ± 0.23
	Clostridia	*Clostridiales*	*o__Clostridiales; f_*	0.00 ± 0.00	14.03 ± 11.82	0.35 ± 0.38	0.73 ± 0.88	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.09 ± 0.17	0.00	9.55 ± 0.44
	Clostridia	*Clostridiales*	*Clostridiaceae*	17.62 ± 15.27	1.28 ± 2.05	1.53 ± 1.92	0.35 ± 0.76	20.81 ± 16.04	0.45 ± 0.90	6.89 ± 9.43	0.22 ± 0.22	9.22	0.26 ± 0.05
	Clostridia	*Clostridiales*	*Lachnospiraceae*	36.12 ± 18.98	21.00 ± 8.50	37.39 ± 9.01	33.05 ± 15.99	7.06 ± 8.29	39.03 ± 14.50	13.89 ± 12.07	27.5 ± 6.86	6.16	27.39 ± 1.31
	Clostridia	*Clostridiales*	*Ruminococcaceae*	16.22 ± 7.65	45.59 ± 15.03	8.79 ± 7.48	25.54 ± 17.03	0.03 ± 0.04	0.38 ± 0.33	0.06 ± 0.06	7.11 ± 6.55	19.05	41.85 ± 0.82
	Clostridia	*Clostridiales*	*Veillonellaceae*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	8.66	0.00 ± 0.00
	Fusobacteriia	*Fusobacteriales*	*Fusobacteriaceae*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ±0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	9.12	0.00 ± 0.00
	Betaproteobacteria	*Burkholderiales*	*Alcaligenaceae*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	9.69	0.00 ± 0.00

Gammaproteobacteria	*Enterobacteriales*	*Enterobacteriaceae*	22.65 ± 18.07	12.71± 13.94	40.37 ± 21.49	24.78 ± 22.35	65.19 ± 11.62	58.91 ± 14.95	77.07 ± 11.11	62.21 ± 11.18	0.98	9.00 ± 0.25

MetaCOMET ANALYSIS

For each time point (3 d.p.h and 7 d.p.h), the microbiota taxa assigned per treatment group were compared in order to reveal a core microbiome, and potentially highlight those features that could be correlated with a phenotype of reduced sensitivity to *Campylobacter jejuni* infection (Figure 75).

3 days post-hatch

Initially, taxonomic composition of 3 d.p.h treatment group samples were compared, both with each other and with CMT and Aviguard® inoculum materials (Figure 76). Looking first at the ASVs shared only between inoculum and each treatment group (not being present in any other microbiota group), CMT and Aviguard® inoculum shared most common features with their respective treatment group (Figure 76a and b). CMT inoculum shared 100 ASVs with 3 d.p.h CMT, 1 ASV with 3 d.p.h Aviguard®, 3 ASVs with 3 d.p.h Internal control and 0 ASVs with 3 d.p.h External control microbiota. Aviguard® inoculum shared 0 ASVs with 3 d.p.h CMT, 27 ASVs with 3 d.p.h Aviguard®, 1 ASV with 3 d.p.h internal control and 2 ASVs with 3 d.p.h external control microbiota. As such, 25.51 % of the ASVs found in CMT inoculum were found only in 3 d.p.h CMT microbiota and 29.35 % of the ASVs found in Aviguard® inoculum was found only in 3 d.p.h Aviguard® microbiota. The bacterial communities of each of the four 3 d.p.h treatment groups were compared in order to reveal a core microbiome of 6 common ASVs shared between all treatment groups, with taxonomic classification of these ASVs being provided in Table 30.

Table 30. Conserved ASV sequences found within the caecal content of birds from all four treatment groups at 3 d.p.h.

			Taxonomy			
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Shigella*	
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*		
Bacteria	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Enterococcaceae*	*Enterococcus*	
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*[Ruminococcus]*	_
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*		
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*			

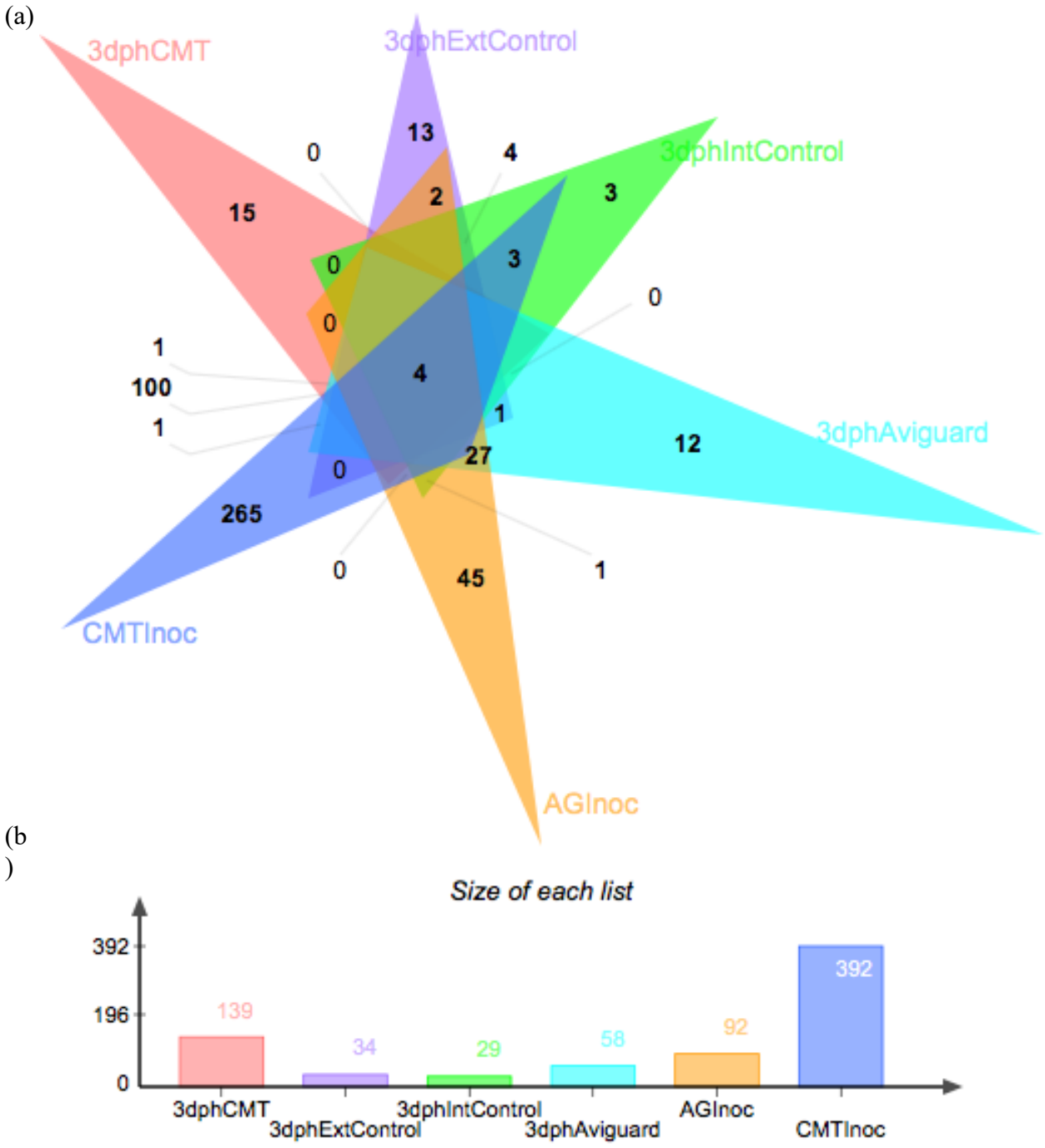


Figure 75. Venn diagram showing ASV sequences (a) shared between specific treatment groups (b) specific to each treatment group from samples collected 3 d.p.h (Aviguard n=3; Ext. control n=3; Int. control n=3; CMT n=4). CMTInoc (n=2) denotes CMT inoculum samples while AGInoc (n=1) denotes Aviguard inoculum samples.

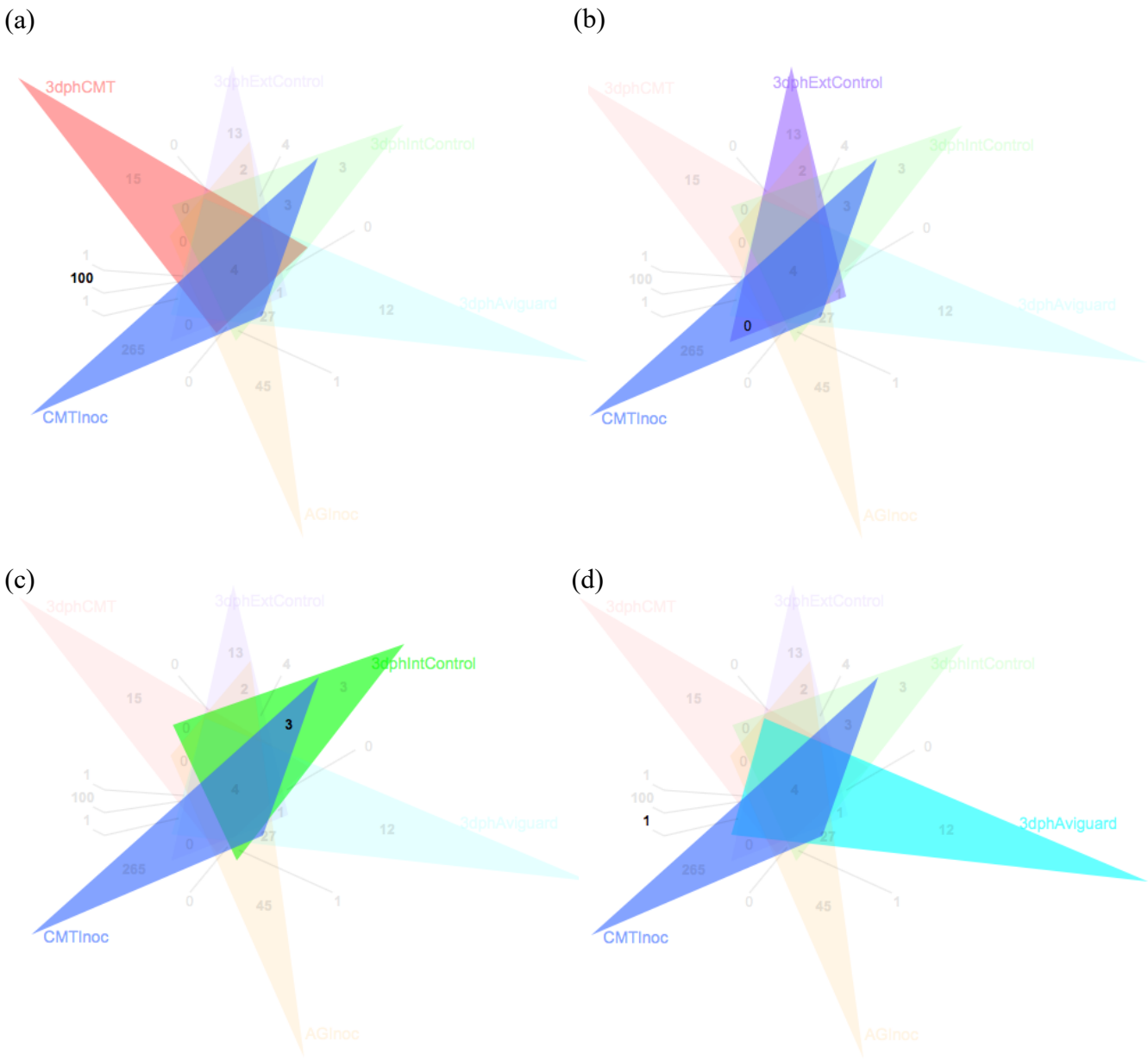


Figure 76. Venn diagrams with highlighted sections showing the number of ASVs shared solely between the corresponding groups. 3 d.p.h treatment groups (a) CMT treated (n=4), (b) Ext. Control (n=3), (c) Int. Control (n=3) and (d) Aviguard® (n=3) directly compared with CMT inoculum (n=2).

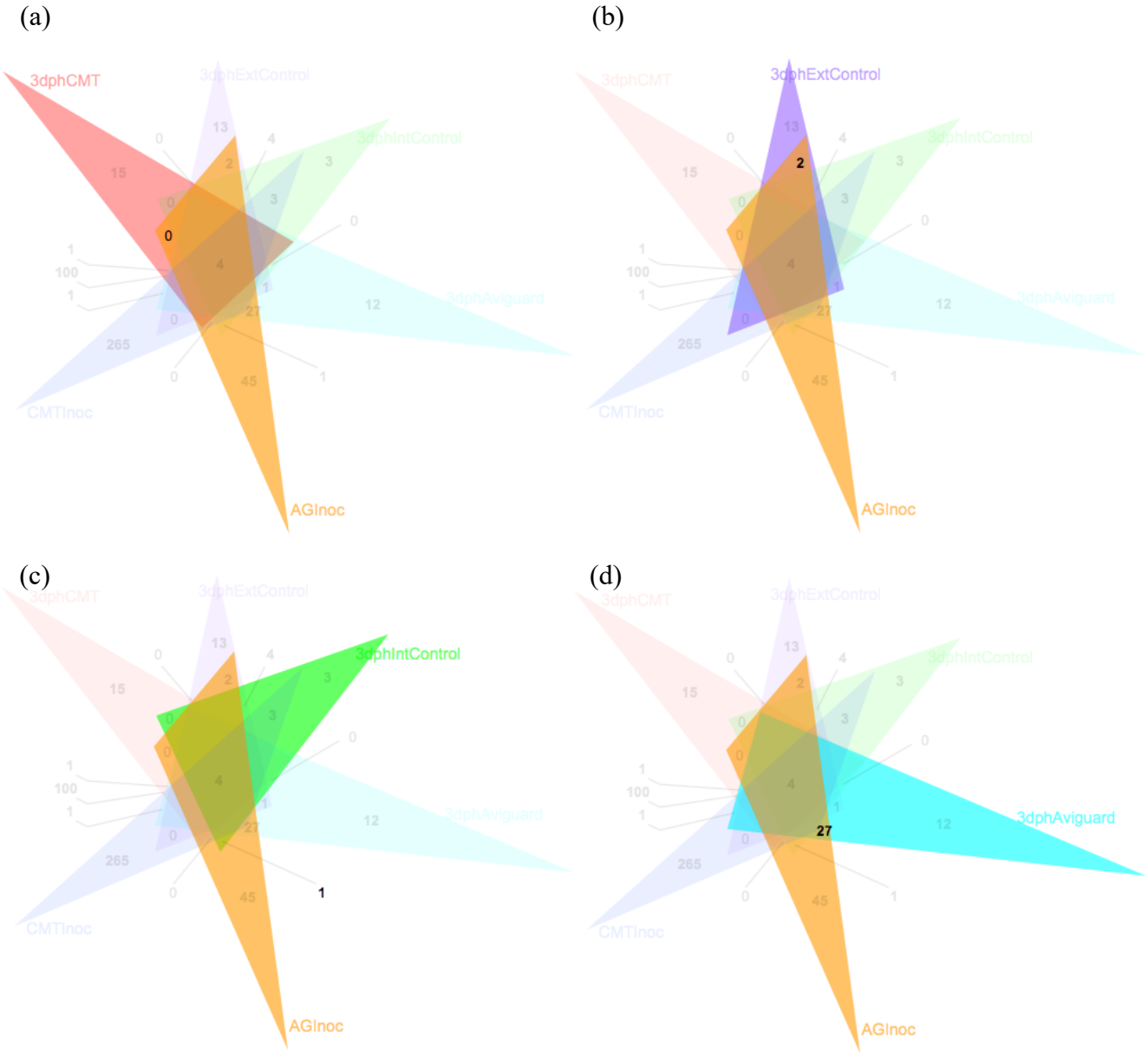


Figure 77. Venn diagrams with highlighted sections showing the number of ASVs shared solely between the corresponding groups. 3 d.p.h treatment groups (a) CMT treated (n=4), (b) Ext. Control (n=3), (c) Int. Control (n=3) and (d) Aviguard® (n=3) directly compared with Aviguard® inoculum.

7 days post-hatch

Similar to that of 3 d.p.h microbiota, 7 d.p.h treatment group microbiota community structure was compared between treatment group and to that of the CMT and Aviguard® inoculum (Figure 78). Looking first at the ASVs shared only between inoculum and each treatment group (not being present in any other microbiota group), as with 3 d.p.h microbiota, CMT and Aviguard® inoculum shared most common features with their respective treatment group (Figure 79; Figure 80). CMT inoculum shared 203 ASVs with 7 d.p.h CMT, 2 ASVs with 7 d.p.h Aviguard®, 0 ASVs with 7 d.p.h Internal control and 5 ASVs with 7 d.p.h External control microbiota. Aviguard® inoculum shared 2 ASVs with 7 d.p.h CMT, 23 ASVs with 7 d.p.h Aviguard®, 0 ASV with 7 d.p.h Internal control and 0 ASVs with 7 d.p.h External control microbiota. As such, 51.78 % of the ASVs found in CMT inoculum were found only in 7 d.p.h CMT microbiota and 25.00 % of the ASVs found in Aviguard® inoculum was found only in 7 d.p.h Aviguard® microbiota. The bacterial communities of each of the four 7 d.p.h treatment groups were compared in order to reveal a core microbiome of 15 ASVs shared between all samples of these treatment groups, taxonomic classification of these ASVs is provided in Table 31.

Table 31. Conserved ASV sequences found within the caecal content of birds from all four treatment groups at 7 d.p.h

Taxonomy						
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Clostridium*	
Bacteria	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Pediococcus*	
Bacteria	*Cyanobacteria*	*Chloroplast*	*Streptophyta*	_	_	_
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rickettsiales*	*mitochondria*		
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	*celatum*
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	_	_	
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*		
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Shigella*	
Bacteria	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Enterococcaceae*		
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Oscillospira*	_
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	_	_
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*[Ruminococcus]*	_
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Clostridium*	
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptostreptococcaceae*	_	_
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*		

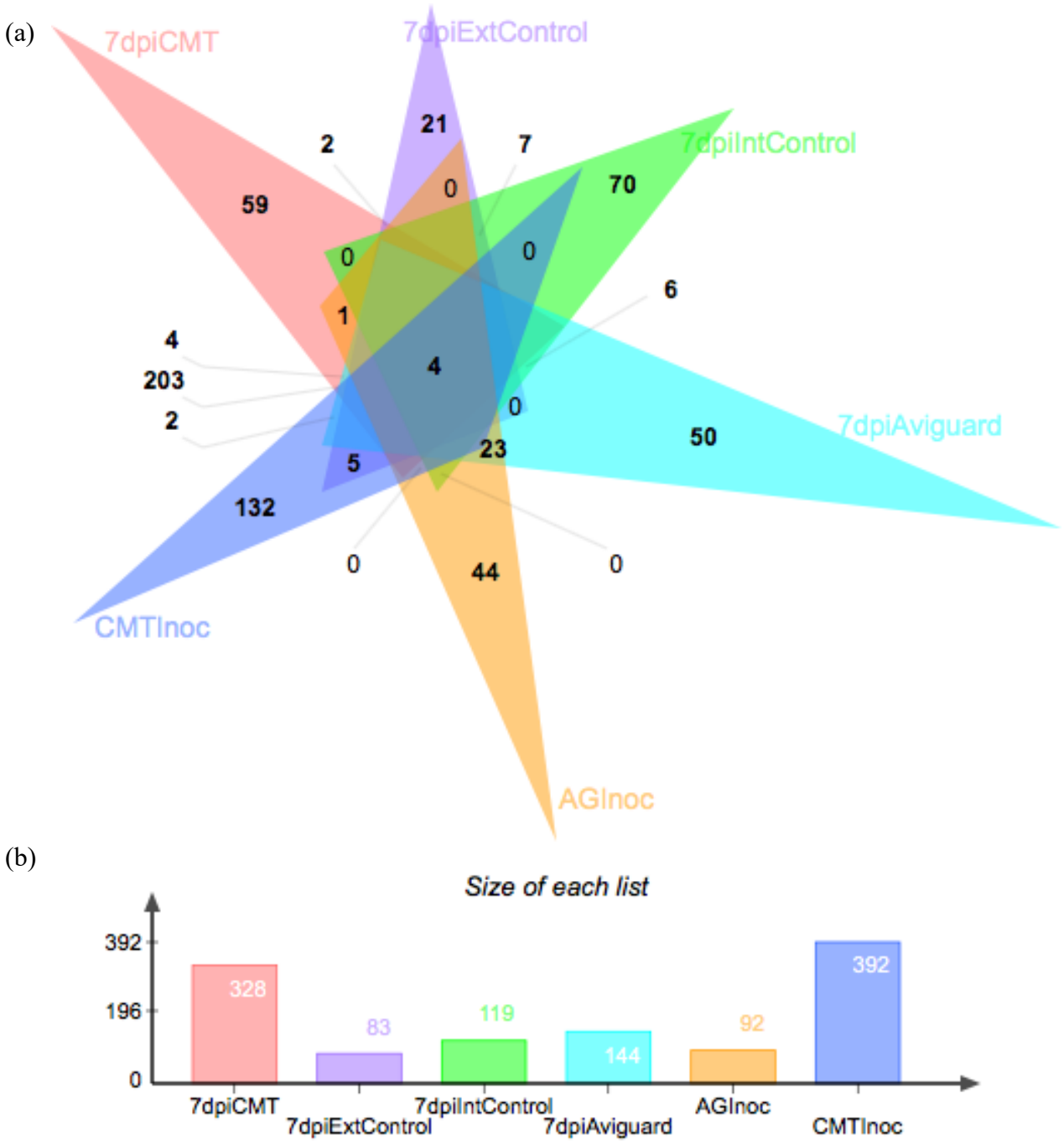


Figure 78. Venn diagram showing ASV sequences (a) shared between specific treatment groups (b) specific to each treatment group (all treatment groups n=10) from samples collected 7 d.p.h. CMTInoc (n=2) denotes CMT inoculum samples while AGInoc (n=1) denotes Aviguard inoculum samples.

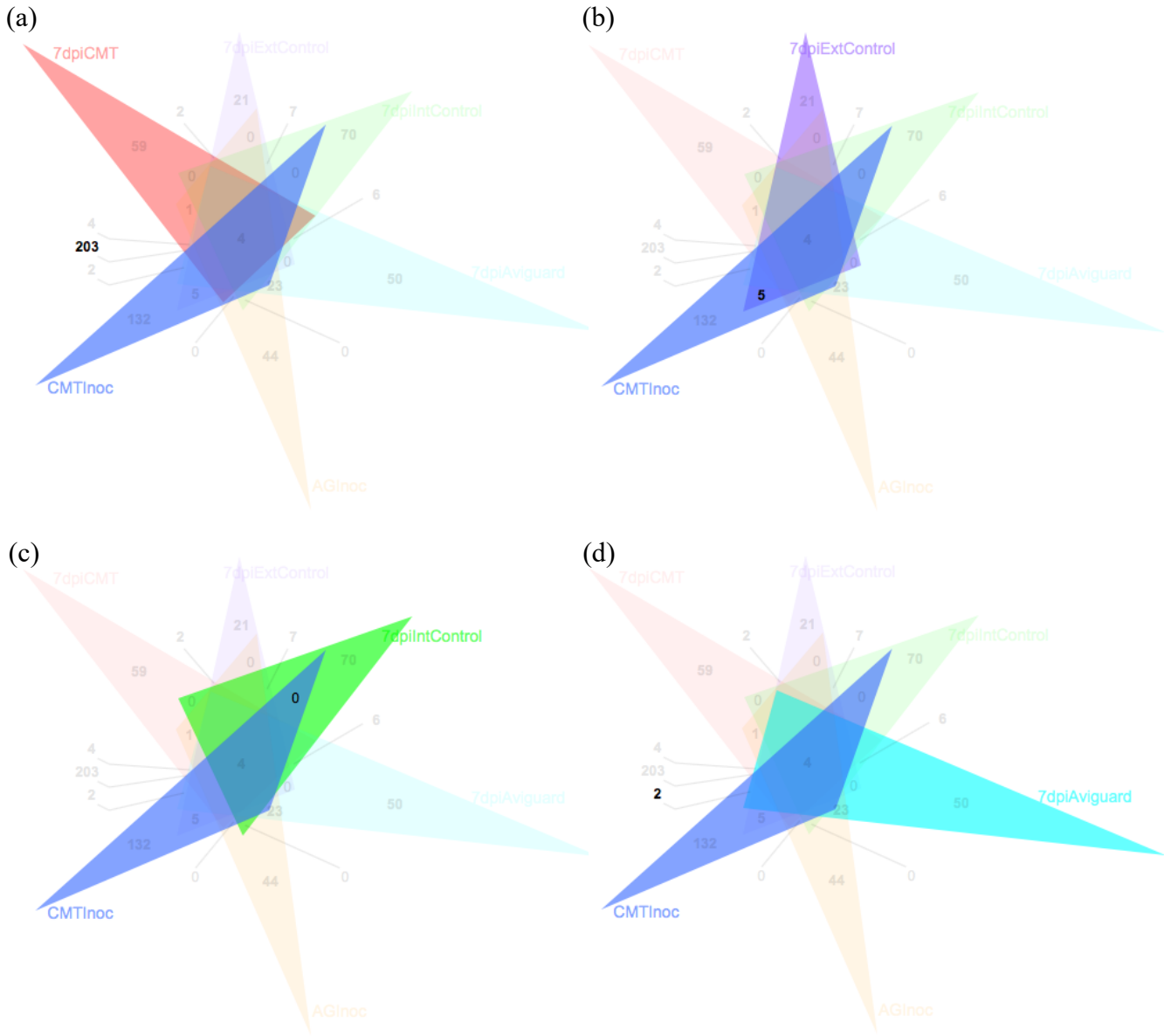


Figure 79. Venn diagrams with highlighted sections showing the number of ASVs shared solely between the corresponding groups. 7 d.p.h treatment groups (a) CMT treated, (b) Ext. Control, (c) Int. Control and (d) Aviguard® treated (all treatment groups n=10) were directly compared with CMT inoculum (n=2)_.

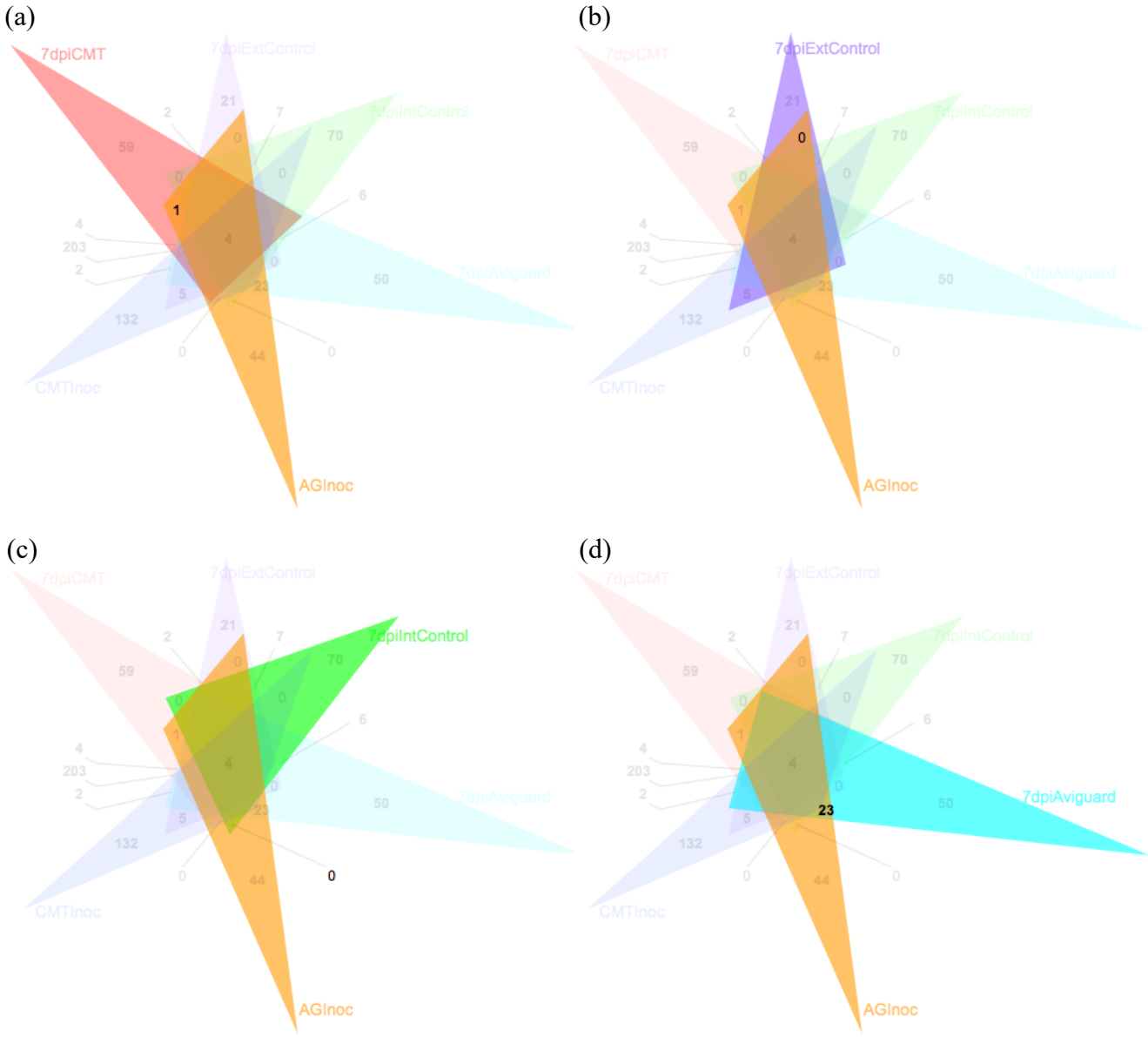


Figure 80. Venn diagrams with highlighted sections showing the number of ASVs shared solely between the corresponding groups. 7 d.p.h treatment groups (a) CMT treated, (b) Ext. Control, (c) Int. Control and (d) Aviguard® treated (all treatment groups n=10) were directly compared with Aviguard® inoculum (n=1).

LEfSe ANALYSIS

To identify which of the ASVs identified may have been differentially represented between our sample treatment groups, LEfse differential abundance analysis was applied. Samples of CMT and Aviguard® inoculum were not included in this analysis because we expected a lot of ASV changes between inoculum material and treatment group samples. LEfse was applied separately to 3 d.p.i and 7 d.p.i treatment group microbiota at family taxonomic level. While no differentially represented taxa were identified in 3 d.p.h treatment group microbiota, 7 d.p.h microbiota analysis returned one bacterial family as being a discriminative biomarker - *Ruminococcaceae* ($p < 0.05$) (Figure 81). *Ruminococcaceae* was significantly more abundant in 7 d.p.h CMT microbiota compared to other 7 d.p.h microbiota communities.

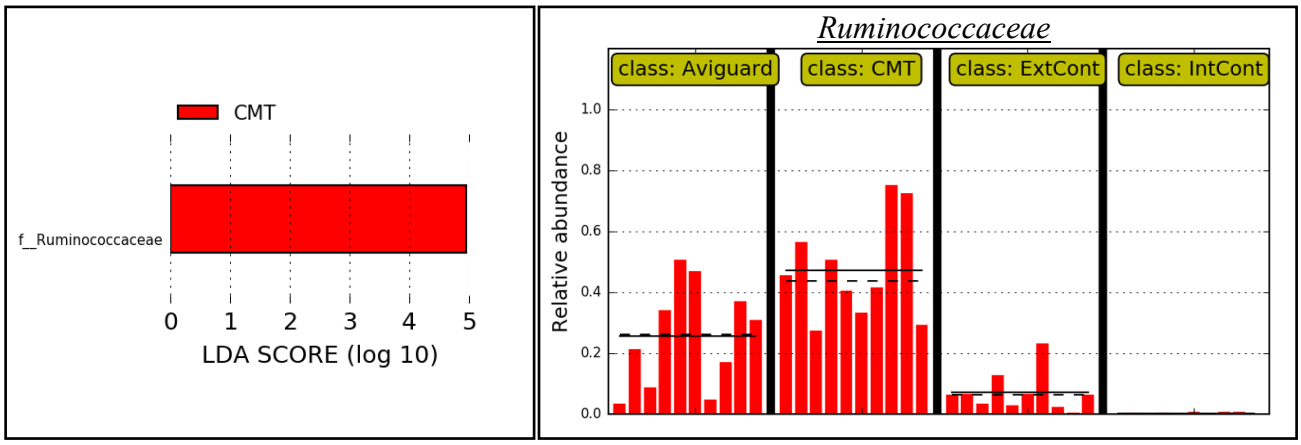


Figure 81. LEfSe results for 7 d.p.h chicken caecal samples (a) histogram of LDA score (log_{10}) derived for biomarkers differentially expressed between treatment groups, (b) Relative abundance data for the detected biomarker with mean and median relative abundance indicated by solid and dashed lines respectively

DISCUSSION

Having exhibited in Chapters 3 and 4 that CMT inoculation has the potential to hinder *C. jejuni* infection of experimental broiler chickens, it was important to develop a mechanistic understanding of the biological rationales underlying this. Bacterial competitive exclusion within the GIT presents an effective approach in limiting pathogenic infection of live poultry in an array of published literature (Nakamura et al. 2002; Al-Zenki et al. 2009; Nurmi & Rantala 1973), examining the microbial communities of our experimental chickens posed an interesting line of scientific enquiry. Caecal samples were selected for all downstream analysis as oppose to other commonly used faecal and ileal samples. Microbial analysis of faecal material has been reported to give less accurate GIT representation compared to caecal microbiota, with the caeca also being the primary site for *C. jejuni* colonisation and harbouring the highest microbial cell density of the chicken GIT (Oakley et al., 2014). High representation of caecal sampling within avian microbiome studies allows this research to be timely and comparable to much of that publicly available (Seargent et al., 2004; Zhu &joergen, 2002).

When considering bacterial communities of the chicken microbiota, age is one of the most influential factors in the shifting community composition over time (Richards et al., 2019; Shang et al., 2018). Although 3-day old chicks treated with CMT inoculum showed generally higher ASV counts, there was no significant difference in microbial richness between the caecal microbiota according to treatment group communities at this early age. While chicken microbial communities are established as early as one day of age, GIT microbial richness is widely shown to increase during the first weeks of life (Kers et al., 2018; Oakley et al., 2014). The increased ASV count for 3-day old Aviguard®, but more strikingly CMT, treated groups may be relatively insignificant in phenotype within the transient 3-day early microbiome but could be essential in driving successional microbiota composition changes establishing a prematurely stable microbiota.

By 7 days of age, Aviguard® and CMT treatment had significantly altered the microbial taxonomic composition of the chick microbiota compared to that of their Internal and External control counterparts. This shift from an intestinal microbiome of low diversity to a more complex and diverse microbiome in the first week post hatch has previously been identified by Ballou et al. (2016) when identifying how early exposures of a chick can later influence development of the chick microbiome. Although microbial community diversity increased for all groups from 3 d.p.h to 7 d.p.h, this was significantly increased in the caecal content of CMT

and Aviguard® treated birds compared to all other groups. While also showing significant increase in microbial diversity, these taxons were most evenly distributed within the samples of 7 d.p.h CMT treated chicks.

Having already identified an increase in bacterial diversity following introduction of complex microflora preparations, be it Aviguard® or CMT, it was important to identify the taxonomic groups underlying this shift in caecal microbial community. A wide source of literature presents early chick microbiota as being primarily *Proteobacteria* Phyla (encompassing *Betaproteobacteria* and *Gammaproteobacteria* at Class level), with the lesser addition of *Bacteroidetes* (Class *Bacteroidia*) and *Firmicutes* (Class *Clostridia* and *Bacilli*) (Ballou et al., 2016; Shang et al., 2018). The function of this early compositional characteristic is still undefined. Successional changes in the gut microbiome of a chick as it ages shows an increase in *Firmicutes* (Kers et al., 2018; Ocejo et al., 2019; Richards et al., 2019), namely of *Clostridia* taxons, with this being appreciably larger in adult chickens. *Bacteroides* and *Preoteobacteria* decrease in the relative composition of adult caecal microbiota while *Actinobacteria* taxons show steady incline (Oakley et al., 2014). Microbiota from both 7 d.p.h CMT and Aviguard® treated chicks principally consisted of *Firmicute*s taxons, compared to that of 7 d.p.h non-treated Internal and External control groups. Increased *Firmicutes* representation, particularly evident in CMT treated birds, could further indicate how the introduction of a complex microbiota has potentially induced ecological primary succession to for a stable, less naïve microbial community in these groups.

Collected from the caeca of 7-week old chickens, the CMT inoculum material was almost exclusively composed of *Firmicutes* taxons. Comparing CMT inoculum with that of 7 d.p.h CMT treated chick microbiota, composition of bacterial taxonomic classes was somewhat comparable between the two communities. In their work on broiler chicken commensal microbial communities, Lan et al. (2005) state how microbial communities are often fully established from 6 - 7 weeks of age within the growing broiler chicken, and so the CMT microbial communities transplanted from these adult chickens to newly hatched chicks represented what should be a relatively stable 'adult' chicken microbiome. Moreover, Kubasova et al. (2019) showed, while investigating the effects of contact between newly hatched chicks and adult hens that chicks raised in the presence of adult birds quickly developed a caecal microbial community similar to that of the adult hens within a week of contact. This understanding is further corroborated by performing compositional analysis of

the microbiota from both 7 d.p.h Internal and External control groups. With neither having received microbial stimulation from a more complex external microbial inoculum, both control groups showed caecal microbial compositions of large *Proteobacteria* abundance.

Although similarly being comprised predominantly of *Firmicutes*, the microbiota from birds treated with Aviguard® inoculum shows comparably lower *Firmicute*s abundance than that of CMT treated chicks, a shift associated concomitantly with an increase in *Actinobacteria* and *Proteobacteria*. Work from Kubasova et al. (2019) reports analogous enrichment of *Actinobacteria*, with the addition of *Bacteroidetes* in Aviguard® treated chicks. Although Aviguard® represents a complex community of bacteria, it presents little in the way of 'natural' complexity compared to that of primary CMT inoculum material. Compositionally, *Firmicutes* are represented to an extent almost half of that in CMT inoculum, with *Bacteroidetes* and *Proteobacteria* comprising a significant proportion of the remaining inoculum material. With these bacterial classes largely associated with a more 'naÏve' chicken microbiota, potential notion could be applied that Aviguard® treated microbiota encompasses a richness and diversity more developed than that of the both control groups but less sophisticated than that of birds receiving CMT inoculum. It can also be postulated that, since Aviguard® inoculum shared only 25.00 % of its taxonomic composition solely with its respective treatment group, compared to 51.78 % for that of CMT inoculum, a large proportion of the Aviguard® inoculum may not be able to form an established and preserved microbial community when transferred directly to a live chicken. As such, the potential environmental adaptation during *ex-vivo* fermentation may alter the expressed phenotype within the avian gut environment and potentially limit the beneficial impact of Aviguard®, and similarly derived competitive exclusion formulations.

Recalling the dissimilarities observed in the microbiota of chicks receiving prophylactic CMT and untreated control chicks, the potential implication of this microbial shift could be related back to the susceptibility of our experimental treatment groups to subsequent *C. jejuni* infection as described in previous chapters. With reduced susceptibility to *C. jejuni* colonisation and transmission observed in CMT treated birds within this project, it is plausible to infer that the change in microbiome resulting from at-hatch CMT inoculation could be one explanatory factor for this reduction in infection and subsequent transmission. While still poorly understood, the influence of CE is widely heralded as one explanation of CMT action and, as noted by Lan et al. (2005), encompasses a wide succession of mechanisms. The

ecological phenomenon of colonisation resistance is a well-known CE mechanism and could be applied to the disrupted colonisation dynamics of *C. jejuni* in birds treated with CMT. Pickard et al. (2017) describes how formation of a stable gut microbiota may inhibit the subsequent invasion and expansion of potentially pathogenic bacterial taxa. With this work going on to connect an immature, infantile bacterial community with reduced protective ability, associations can be made between the naïve microbiome of our experiment control, and to a certain extent Aviguard®, chicks and their increased susceptibility to *C. jejuni* infection compared to chicks receiving CMT (Pickard et al., 2017). This may also prove to exemplify how it is critical for CMT intervention strategies to be applied immediately post hatch to ensure establishment of the desired microbial community. Due to the vast complexity of interactions within the GIT microbiome alongside host-microbiome interactions, unearthing the exact rationale behind this protective effect is still to be achieved, however, direct competition for the occupation of both physical and nutritional niches has been postulated (Pickard et al., 2017). Persistent *Campylobacter* infection of the avian intestinal tract is heavily reliant on interaction with the avian intestinal mucosa (Hermans et al., 2014). Administration and subsequent establishment of a complex bacterial community to a newly hatched chick offers commensal adherent bacteria a distinct advantage in colonisation of the gut mucosal surface, forming a microflora that already occupies the adhering niches essential during later *C. jejuni* infection (Lan et al., 2005). This competition for adhesion site is often described in conjunction with competition from nutrient and physical space within the microbiota, all being likely confounding factors that can be applied here (Lan et al., 2005).

Upon entry to the CMT enriched GIT, invading *C. jejuni* will not only have to encounter increased adhesion site competition from an established, native, microbial community but also potentially inhibitory metabolites produced by the bacterial taxa of such a community. From the bacterial community that comprises the broiler chicken microbiota, one of the most notable terminal metabolites produced is butyrate (Onrust et al., 2015). Butyrate is a SCFA produced in the GIT during bacterial digestion of carbohydrates and dietary fibre (Guilmeau et al., 2013). Having been identified for beneficially affecting chicken growth and intestinal integrity following dietary supplementation, intestinal butyrate has long since been linked with the control of important avian pathogens including *Salmonella Enteritidis* (Onrust et al., 2015) and enhancement of anti-inflammatory properties. Increasing in caecal dominance with increasing bird age, *Firmicutes* commonly comprise over 80% of the adult caecal microbiota (Onrust et al., 2015). Of this prominent bacterial group, various bacterial Families, including

Lachnospiraceae and *Ruminococcaceae* are known efficient butyrate producers (Onrust et al., 2015). When present in a sufficiently high concentration, Van Deun et al., (2008) has shown butyrate to have a strong bactericidal effect on *C. jejuni in-vitro*. Coupled with the benefit to intestinal epithelial structure and anti-inflammatory influence, a similar inhibition of butyrate on *C. jejuni* may be observed as that long described for S*almonella* (Nurmi & Rantala 1973). Analysis of the microbiota of our experimental chickens following CMT treatment shows significantly increased presence of the butyrate producing *Ruminococcaceae* family taxons to levels of that seen in CMT inoculum from adult chickens. This leads to the inference that CMT inoculation develops the intestinal microbiota, creating a caecal environment highly rich in butyrate and, as a result, considerably less hospitable for the invading *C. jejuni*.

In the absence of experimental microbial transfer at hatch, *Proteobacteria* taxons continued to dominate the microbial communities of 7-day old chicks. Of the *Proteobacterial* Phyla, *Enterobacteriaceae* was almost the sole bacterial Class in our treatment group microbiota. Interestingly, Winter et al., (2014) has explored the impact of *Enterobacteriaceae* on the human GIT, relating an expansion in taxa abundance of this facultative anaerobe to intestinal dysbiosis. Additionally, a bloom of *Enterobacteriaceae* is commonly a sequential event evolving from enteric pathogenic infection in mice. In relation to the broiler chicken, the FSA has postulated that the *Enterobacteriaceae* may be used as a crude indicator of broiler carcass *Campylobacter* contamination, with levels of intestinal contamination of both bacterial taxa being analogous (Corry et al., 2017). Corroborated by Sakaridis et al., (2018), there is an undeniable correlation between high *Campylobacter* colonisation and an increased relative abundance of *Enterobacteriaceae* in the avian caeca. This same research raised an important theory on the temporality of causation, in that *Enterobacteriaceae* may create an intestinal environment beneficial for *Campylobacter*, or v*ice versa*. With our microbiota samples showing a clear indication that higher *Enterobacteriaceae* abundance, shown here in both Internal and External control treatment groups, might be associated with subsequent susceptibility to *Campylobacter* infection, this research may go some-way in elucidating a resolution (Sakaridis et al., 2018).

It is undeniably clear from this research that the intestinal ecosystem of the broiler caecum contains a vast and diverse bacterial community, with each taxon performing different functions to influence host growth, performance and susceptibility to infection. The dynamic relationships between particular bacterial taxa and a predisposition to *Campylobacter*

susceptibility proves that manipulation of the caecal microbiome is an essential facet in any *C. jejuni* control strategies. Not only may a CMT rapidly and prematurely develop the avian microbiome, it may also outcompete currently available competitive exclusion products through the introduction of an 'adult', complex microbiome not achievable through their *ex-vivo* culture. Future research might place interest in the intriguing portion of unclassified bacterial taxa observed primarily within both CMT inoculum and the microbiota of CMT treated chicks at 7 d.p.h. A complex community of remarkable bacterial abundance elucidating the complete catalogue of components inhabiting the chicken microbiota remains elusive. As such, a large portion of unknown bacterial taxa, often referred to as 'microbial dark matter' continues to form the backbone of microbiome research in both human and veterinary models. A current limitation in the use of reference-based analysis of next generation sequencing output is the fundamental lack of 'completeness' in even the most comprehensive of reference catalogues. As such, these techniques are largely constrained by the databases available at the time of data analysis which may result in the oversight of taxonomic groups with crucial biological importance. Such a limitation is acknowledged within the research presented here. Further to this, 16S rRNA gene sequencing techniques have long been hampered by introduction of biases from PCR artefacts such as, formation of chimeras and preferential amplification of specific bacterial taxa over other (Sze & schloss, 2019). While little can be done to limit such factors, implementation of whole genome sequencing as a progression of this work would go some way in minimising the effect on our data set. We would also gain vital functional insight into these bacterial groups, alongside profiling of archaeal, viral, protozoan and fungal communities not assessed here.

In addition to the bead-beating techniques and specific selection of DNA extraction kit used here, all future work should incorporate the use of a well-defined microbial standard community. These mock communities of gram-negative and gram-positive bacterial communities allow for more precise optimisation and validation of cell lysis processes during standard DNA extraction.

Chapter Six: General Discussion

GENERAL DISCUSSION

Absence of obvious pathological clinical indication makes *C. jejuni* infection within commercial poultry of limited animal welfare concern (Alpigiani et al., 2017). However, its continued establishment as a causative agent for global human campylobacteriosis has made *C. jejuni* a persistent risk to public health (Rushton et al., 2019). This project aimed to build on the current understanding of *Campylobacter* infection within the broiler chicken to gain further insight into the infection biology of *Campylobacter* alongside the long-term Immune reaction to infection within the commercial broiler chicken. Using this understanding we aimed to assess the efficacy of the otherwise unexplored concept of caecal transplantation within the broiler chicken in reducing *Campylobacter* infection at flock level.

Since the immune response to *Campylobacter* infection within the broiler chicken is yet to be fully defined, a multifaceted approach to its study throughout Chapter 2 was employed, utilising both bacteriological and immunological techniques. From point-of-inoculation at 3 weeks post-hatch, colonisation of the intestinal tract with *C. jejuni* was that of rapid and sustained establishment until common point-of-slaughter, largely reflecting that described in work by (Humphrey et al., 2014; Smith et al., 2008). This work provides support to the generally accepted notion of *C. jejuni* colonisation showing greatest burden within the caecal crypts, but sporadic presence elsewhere within the GIT (Hermans et al., 2011). Median bacterial loads at point of slaughter exceeding 7.6 log CFU per g caecal content in both studies of Chapter 2, and as such, would represent a significant source of contaminating *Campylobacter* cells to both the individual and associated flock broiler carcasses during slaughter and processing (Berrang et al., 2004). While no chickens associated with this study showed any signs of malaise, recovery of *C. jejuni* from systemic tissues of the chicken within both experimental studies of Chapter 2 strongly support earlier indication that *C. jejuni* is able to traverse the intestinal mucosal barrier and cause systemic infection. This systemic *C. jejuni* propagation into tissues distant from the intestinal tract, including blood, lung, spleen, liver and heart has been reported throughout available literature sources (Knudsen et al., 2006; Cox et al., 2006; Young et al., 1999). While this area of research has an underlying dogma associated with a lack of definitive description of *C. jejuni* translocation across the intestinal epithelial barrier, a number of theories are continually identified in published literature. The majority of scientific reports centre upon *C. jejuni*-induced intestinal epithelial barrier disruption – particularly due to alterations of in the proteins forming tight-junction barriers (Awad et al., 2013). This is often associated with prolonged *C. jejuni* co-incubation and as such,

has been described for later stages of infection, similar to findings seen here. Epithelial barrier permeability could be further exaggerated by findings of Connell et al (2013), who demonstrated cadherin cleavage alteration to adherin cellular junctions. Transcytosis through specialised M cells is a well-known mechanism for passage across the intestinal epithelial barrier for other enteric pathogens, and as such has been linked as a potential mechanism for *C. jejuni* systemic invasion of the chicken (Backert et al 2013). However, reports of such interaction for *C. jejuni* in the chicken are scarce and so the lack of consensus understanding in this field of research remains. This is of particular concern with chicken liver representing a common protein source globally, acting as a poorly described vehicle for human ingestion of *C. jejuni* (Lanier et al., 2018).

Since the understanding that *C. jejuni* is not simply a commensal within the broiler chicken has become ever-more accepted, increasing bodies of research now exist that focus on the immune response of the chicken to infection. However, such work is often restricted to early time points post infection. Using *post-mortem* samples of caecal and caecal tonsil tissues collected throughout the four-week period post-infection, Chapter 2 illustrates the induction of an innate immune response within both tissues. Two days following initial exposure of birds to *C. jejuni*, both tissues exhibited the induction of IL-1β, IL-6, IL-17A and CXCLi2, all known pro-inflammatory signals. Early upregulation of such transcripts is also illustrated in the findings of Humphrey et al., 2014 and Smith et al., 2008. Elevation of these innate pro-inflammatory regulators remains until 14 days post infection within caecal tissues, however, this is accompanied by upregulation of signals associated with regulatory ($TGF\beta_4$) and Th2 (IL-4) responses from as early as 7 days post challenge. By 7 days post challenge, caecal tonsil tissues showed upregulation of only mediators, with the addition of IL-10. While IL-10 and $TGF\beta_4$ play central roles in regulating the inflammatory response to infection through T regulatory lymphocyte (Treg) stimulation, IL-4 is a potent mediator in humoral immunity (Humphrey et al., 2014). As such, it might be postulated that the immunoglobulin stimulation seen within serum IgY and IgM of our experimental studies might be driven by this IL-4 expression. These immunological findings alongside associated *in vivo* infection phenotype, provide a valuable understanding on how the avian immune system responds to *Campylobacter* infection, and forms a framework supplementing other research from which a therapeutic agent to control infection might be developed.

Common to all *in vivo* studies within this project, inoculation of experimental animals was conducted at 21 days post-hatch. Employment of such design was largely on the basis of understanding that prevalence of *Campylobacter* infection within the commercial broiler flock incurs an age-dependent lag phase of 14 – 21 days post-hatch (Stern et al., 2001; Conlan et al., 2007; Conlan et al., 2011). Use of this administration time-point minimised the potential influence of these lag-phase determinants on the infection biology characteristics observed and is a protocol previously described in literature (Humphrey et al., 2014; Chaloner et al., 2014). In itself, this ecological characteristic of *Campylobacter* infection within the chicken has been hailed as a 'window of opportunity' for the implementation of intervention strategies aimed at reducing or eliminating *C. jejuni* burden within the commercial poultry flock.

While countless control strategies have been postulated to mitigate *Campylobacter* infection within the commercial broiler chicken, there remains no effective method of intervention in place (Lin., 2009). Conceivably the most important factor associated with the limitation of human exposure to *Campylobacter* infection would be to minimise broiler carcass contamination, with Meunier et al., 2016 highlighting colonisation at primary production as crucial to this. This production stage can be further distinguished into two successional points at which intervention strategies could be applied, those acting as preventative measures which aim to reduce likelihood of initial colonisation and those acting as colonisation reduction methods that aim to reduce or eliminate an already established infection (Ghareeb et al., 2012). Derived from this understanding, multiple approaches to *Campylobacter* control on the commercial broiler farm have been suggested and assessed, with the rationale and efficacy of each being described in Chapter 1. While increases in on-farm biosecurity practises has long been heralded as an effective control of *Campylobacter* within the poultry industry, recent understanding is that this intervention may have reached saturation point in regard to efficacy without further understanding of risk factors associated with infection (Lin., 2009). Although of a different nature, vaccination strategies are accompanied by their own associated limitations, with the restricted window of functional application within the commercial broiler chicken largely hampering commercial realisation (Pasquali et al., 2011).

As our understanding of the importance of the intestinal microbiota on the health and welfare of its host, increasing interest is being placed onto the use of prophylactic microflora cultures as an antibiotic-free medium in preventing or reducing the frequency of *Campylobacter* incidence (Johnson et al., 2017). Microbial therapies showing generally lower taxonomic

complexity, such as probiotic preparations have been developed in abundance, however their effectiveness within the poultry industry remains controversial (Clavijo & Flórez, 2018). With relatively little in the way of taxonomic diversity, these products show tendency toward only transient modification of the host microbiota only in the periods after consumption (Ciorba., 2012). Of conceivably more benefit are complex microflora products, with these being either Competitive Exclusion (CE) products, or the introduction of entire donor microbiota through Faecal Microbiota Transplantation (FMT) procedures. While CE products such as Aviguard® have long been used in poultry production, with mixed success, FMT has largely been confined to human based application in the treatment of Clostridium difficile infection (Aas et al., 2003; Agrawal et al., 2016).While the entirety of the avian gastrointestinal tract comprises an array of microbial taxonomies and communities, it is the caecal crypts that contribute greatest to this bacterial abundance, while also being the primary site of *C. jejuni* colonisation. As such, for this project, Caecal Microbiota Transplantation (CMT) procedures replaced those of the more commonly known FMT, based on the understanding the both concept and function is will be largely transferable.

Until recently, FMT use in both human and veterinary medicine was not subject to widespread application, and consequently, continues to lack formal guidelines for standard practice of donor and recipient selection, screening of microbiota preparation and administration (Borody & Khoruts, 2012). This is particularly true for use of CMT within poultry, whereby this novel application has previously been overlooked. Using consistent methodologies regarding CMT preparation and administration, this project has been able to produce reproducible protection of broiler chickens against experimental *C. jejuni* infection. Protocols defined here could aid the development of standardised guidelines for the application of CMT within the chicken.

With experimental study replicates of Chapter 3 and Chapter 4 largely confirming potential efficacy of CMT practices in the prevention of *C. jejuni* colonisation and establishment at flock level, we were subsequently able to consider both the comparable level and mechanism of this protection against a commercial CE product in both Chapter 4 and Chapter 5. Phenotypically, prophylactic administration of Aviguard® preparation showed limited efficacy in improving host susceptibility to *C. jejuni* infection, with no obvious reduction in infection frequency, and reduction in colonisation burden against only one control group. While

protective success of Aviguard® has been described throughout research, this is primarily described for *Salmonella* based infection models and as such, limited data exists on the efficacy of such product against *C. jejuni* within the broiler chicken (Hofacre et al., 2000; Ferreira et al., 2003). With this research, we can begin to unearth how Aviguard® preparations might influence the unique infection biology characteristics of *C. jejuni* within the broiler chicken.

Both FMT and CE preparations are, theoretically, used on the basis of introducing a complex microbial environment to the recipient, with the aim of establishing the early colonisation of a 'normal' protective microbiota (Wagner et al., 2006). This is particularly pertinent within the poultry industry, whereby a lack of parental contact post-hatch and general environmental sterility might dampen the development of the early pioneering microbiota (Videnska et al., 2014). Beginning immediately post-hatch, the early development of the chicken microbiome is a dynamic process of rapid successional changes, with bacterial taxonomic composition shifting at an almost daily rate (Gilbert et al., 2010; Juburg et al., 2019). It is during this period post-hatch that the intestinal microbial community is thought to be most susceptible to modulatory interventions (Juburg et al., 2019). *In vivo* data presented in Chapter 3 supports this understanding, with birds receiving CMT administration at 7 days post-hatch showing marked reduced susceptibility to *C. jejuni* infection compared to similar studies whereby CMT had been delivered early post-hatch. With widespread indication that these bacterial colonisation characteristics are primarily the effects of direct or indirect competition, or competitive exclusion, between resident and colonising taxa, Chapter five used 16S ribosomal RNA (rRNA) sequencing tools as a means of uncovering the influence of such theory on the *in vivo* results already attained. With the upsurge in use of Next Generation Sequencing (NGS) tools such as 16S rRNA gene sequencing over the previous decade, an increase in understanding of the compositional dynamics of the chicken microbiome has become evident throughout literature (Richards et al., 2019; Schokker et al 2014; Connerton et al., 2018).

Findings within Chapter 5 for caecal samples obtained 3 days post hatch (3 d.p.h) are supportive of conclusions made in the work of Richards et al., (2019) and Ballou et al., (2016), whereby the early microbiome of the commercial broiler chicken is characterised by a low taxonomic diversity and predominated by *Enterobacteriaceae* (phylum *Proteobacteria*). Considering next the birds of this age treated with either CMT or Aviguard® preparation, both were associated with a caecal composition showing lower *Enterobacteriaceae* contribution

and predomination of *Clostridiales* (phylum *Firmicutes*). Successional development of the caecal bacterial communities by 7 d.p.h of our study had increased *Lachnospiraceae* abundance within the untreated chicken microbiota, with associated decline in *Enterobacteriaceae*. This developmental trait of the avian intestinal microbiome is well characterised within available literature and is thought to continue at similar trend until approximately 14 days post-hatch whereby *Firmicutes* taxons will predominate at over 90 % (Ocejo & Hurtado, 2019; Videnska et al, 2014). While the exact function of this early *Enterobacteriaceae* dominated microbiota remains unclear, Richards et al*., (*2019) confirms its relatively short-lived occurrence, with peak relative abundance between only 0 – 3 days post-hatch. Treatment of chicks with either of the studied microflora preparations (CMT or Aviguard®) appeared to rapidly accelerate this progression away from *Enterobacteriaceae* toward *Firmicutes* taxons, with this being particularly apparent for CMT treated birds. By 7 d.p.h, *Ruminococcaceae* was the predominant taxa within the caecal microbiota of CMT treated birds. With taxonomic establishment of Firmicutes to such high relative abundance usually occurring appreciably later in stages of development within the broiler chicken microbiome, it could be perceived that CMT administration is causing premature maturation of the broiler microbiota to that of an adult chicken. Composition of the caecal content of CMT treated birds was largely emulated in that of the CMT inoculum, a facet not achieved for Aviguard®. This might give further support to the understanding that microflora preparations not achieving the same taxonomic complexities as seen within whole caecal samples may provide only transient microbial modulation.

While much of the protection derived from many microflora preparations is attributed to this competitive exclusion principle, stimulation of the host immune system has also been widely theorised as a supplementary mechanism (Ajuwon, 2015). *In-vitro* models have commonly been utilised as a means of assessing the interactions between pathogen and host (Barrila et al., 2018). While somewhat reductionist in approach, these *in-vitro* models can draw attention to underlying mechanisms that could be conceptually applied as a basis for further in vivo experimental design. Contrary to the adhesion and invasion characteristics identified for *Campylobacter* infection *in vivo*, Chapter 4 states how both *S.* Typhimurium and *C. jejuni* were effectively about to invade avian intestinal epithelial cell monolayers *in-vitro*. This disparity in invasive capabilities of *C. jejuni* to invade avian intestinal cells between *in vivo* and *in-vitro* systems has previously been described Byrne et al., 2007. While pre-treatment of cell monoloayers with CMT filtrate gave significant protection from *C. jejuni* and *S.* Typhimurium

invasion, pre-treatment with Aviguard® filtrate showed only protection against *S*. Typhimurium invasion. While the exact mechanisms behind this effect are not fully understood, the sterile filtration of this inoculation material to remove bacterial molecules would suggest that secreted metabolites, such as short chain fatty acids, from *Lachnospiraceae* and *Ruminococcaceae* show influence on avian epithelial cells and invading pathogenic organisms to prevent infection. Such an influence may be dictated by induction of early pro-inflammatory host responses that, in turn, induce premature immunomodulation through regulatory cytokine cascades. Particular genus of *Rumminococcaceae*, including multiple *Faecalibacterium prausnitzii* strains, experienced limited scientific exploration largely due to difficulty in culture of this oxygen-sensitive species. More recently, human medicine and in turn, veterinary medicine, have dedicated a wealth of investment in understanding *Faecalibacterium prausnitzii* due to its observed anti-inflammatory effects against human colitis (Sokol et al., 2018). Qiu et al (2013) observed that both cellular and supernatant components of *F. prausnitzii* suspensions were are to provide a positive environment for T-reg and IgA production, likely through stimulation of cytokines including TGF-$ß_1$ and IL-10.

While the results seen throughout chapters 3-5 indicate that the at-hatch delivery of an adult chicken microbiome to chick has potential reducing susceptibility to *C. jejuni* infection spanning the life-span of the commercial broiler, consideration must be applied to the safety aspects associated with its application. While this research goes some way in outlining how CMT therapies can be applied to the commercial broiler chicken, and indeed how this might induce stimulatory immune responses and modifications to the intestinal microbiota, there is need for continued refinement before confident large-scale application aimed at reducing susceptibility to *Campylobacter* infection. It should also be noted that, although apparently beneficial in protection against *C. jejuni* colonisation, CMT administration was also correlated with a negative impact on final bird body weight. This finding has not been highlighted in previous probiotic research for the chicken and so may be specifically associated with CMT administration. A list of key project findings is provided in Table 32.

Table 32. Table of key project findings

Conclusion	Reasoning	Location of findings
***C. jejuni* is able to form a rapid and prolonged colonisation of the broiler chicken GIT**	*C. Jejuni* was present within the caecal content from as early as 2 d.p.i	Chapter 2
	Once colonised, high *C. jejuni* load persisted until point of slaughter at 28 d.p.i (49 d.p.h)	
	Identification of C*. jejuni* within ileal regions	Chapters 2, 3 & 4
***C. jejuni* is able to colonise beyond the chicken GIT**	Detection of *C. jejuni* within liver and spleen tissue samples from infected chickens	Chapters 2, 3 & 4
	Invasive ability of *C. jejuni* strains using *in-vitro* avian intestinal cell models	Chapter 4
GIT tissues exhibit early pro-inflammatory and Th-17 responses to *C. jejuni* infection	Early upregulation of IL-1β, IL-6, IL-17A and CXCLi2 in caecal and caecal tonsil tissues during early infection time points (2 d.p.i)	Chapter 2
Prolonged *C. jejuni* challenge causes subsequent induction of regulatory responses	Subsequent induction of IL-10 and TGFβ_4 in caecal and caecal tonsil tissues during later stages of infection - from 7 d.p.i onward	Chapter 2
CMT derived from 'adult' broiler chicken microbiota is effective in reducing susceptibility of broiler chickens to experimental *C. jejuni* infection	Reductions in flock shedding of *C. jejuni* within flocks treated with CMT preparations	Chapters 3 & 4
	Reduced caecal burden of *C. jejuni* within birds of CMT treated flocks	
	Percentage invasion of *C. jejuni* into avian intestinal cell lines *in-vitro* was reduced following prophylactic incubation with CMT filtrate	Chapter 4
Efficacy of CMT treatment is reliant on its administration to an early, naïve chick microbiota	Administration of CMT to chicks at 7 d.p.h marginally disrupted *C. jejuni* flock transmission and showed no influence on caecal burden at 12 d.p.i (33 d.p.h)	Chapter 3
Administration of CMT to newly hatched chicks modulates the caecal microbiota, creating a bacterial community more representative of an 'adult' chicken microbiota	Early caecal content of CMT treated chicks shows considerably higher relative abundance of *Firmicutes* taxons compared to caecal content of untreated chicks, being predominantly *Proteobacteria*	Chapter 5
	Increased representation of *Ruminococcaceae* within the caecal content of CMT treated chicks 7 d.p.h	
	Increased frequencies of observed ASVs within caecal content of CMT treated birds compared to other treatment groups	
CMT shows more taxonomic richness compared to Aviguard® preparation	Increased number of ASVs identified within CMT preparation material compared to that of Aviguard® preparation	Chapter 5
CMT might be better able to cause prolonged modifications to the caecal microbiota than commercial CE products	CMT preparation and caecal content of CMT treated chicks at both 3 & 7 d.p.h showed considerably more uniquely shared ASVs than seen for Aviguard® preparation and Aviguard® treated chicks.	Chapter 5

STUDY LIMITATIONS

Description of study limitations has been provided throughout this thesis in the discussion pertaining to each chapter. Crucial to the reliability of the results gained from *in vivo* experimental protocols, was use of a sufficient number of experimental animals per treatment group. A continued core limitation to this project was the ability to obtain expected treatment group sizes from eggs hatched within our experimental unit. Although it is difficult to ascertain exact causes of the reduced hatchability rates observed within a number of the experimental trials of this project, potential cause could lie with a low egg viability or exposure of eggs to incubation conditions that do not meet those required by the developing embryo. While not directly impacting the outcome of the associated experimental trials, treatment group sizes of lower magnitude might reduce the general power of the study. Confounding this, drawing accurate comparisons between treatment groups of differing sizes allows for introduction of bias into any conclusions drawn. Stemming from this, it would have been of perceivably greater importance to provide larger numbers of samples submitted for 16S rRNA per treatment groups, per sampling timepoint. This would also allow for increased sampling time points between hatch and *C. jejuni* infection potentially unearthing crucial stages in microbiota development. Increased sampling magnitude here could generate datasets better able to identify caecal microbiota alterations associated within specific therapy. Processing of such microbiota samples would also have gained a greater credibility from the inclusion of microbial mock communities to control for potential deleterious effects of DNA extraction techniques on certain bacterial communities.

The ability to accurately assess the immunomodulatory effects of CMT might also be limited by the use of *in vitro* cell line monolayers within this project. While use of 2D cell monolayers are a commonly utilised tool in understanding the potential interactions between host cells and external stimuli, the *in vivo* relevance of these studies remains somewhat restricted. It has often been suggested that these cell models do not comprehend the complexity of living physiological systems and so are unable to accurately reflect a true biological picture (Langhans., 2018). Of further consideration should be the use of a more comprehensively described cell-line, able to provide a more defined limit to cellular responses following

infectious challenge. This would provide the results gained here a greater degree of biological relevance.

Although undeniable that the administration of CMT at point-of-hatch resulted in biological manipulation of the avian intestinal tract, it is conceivable that the processing of CMT material following collection could be modified to reduce potential loss of oxygen-sensitive microbial species. Use of anaerobic workstations and pre-reduced diluent to prepare such material would be a relatively simple method to achieve this.

FURTHER WORK

Experimental data gained within this project provides insight into how the broiler chicken immune system might respond to long-term *Campylobacter* colonisation. To develop in-depth understanding of this complex host-microbe interaction, data such as that obtained here can be collated with previous work focused on early innate responses to generate explanatory statistical models of interdependent immunological systems (Reid et al., 2016).

With 16S rRNa sequencing tools heralding useful insight into how CMT and Aviguard® might influence patterns of microbial taxonomic community assembly within the broiler caecum, this project perhaps lacked further understanding in the functional outcomes of these shifts. Metabolomic tools might provide a useful means of assessing which, if any microbial metabolites might be differential represented within CMT treated birds, how this might be reflective of the caecal microbial community, and finally, how this might influence susceptibility to intestinal disease and dysbiosis. Additionally, the extensive use of whole-genome sequencing as oppose to simply 16S rRNA tools would allow for not only deeper functional insight into the bacterial components of this environment, but also archaeal, fungal, viral and protozoan microbial groups. Using whole genome sequencing in this way would also allow for *de novo* taxonomic assignment, reducing researcher reliance on reference databases and thus allowing for the potential identification of novel species and strains previously overlooked. Such techniques could be applied in a regular fashion following CMT administration to provide a more comprehensive understanding of successional development in the chick microbiota following treatment. Further to this, sequencing in this

manner could also be used to create a more defined catalogue of the exact components found within the CMT inoculum material.

Early work presented throughout this project has provided weight to the reproducible protective effect of CMT against *Campylobacter* within the broiler chicken. However, to allow us to refine its use as a viable prophylactic measure we must understand how we can tailor its use to complement modern day poultry farming practices. Further work into the efficacy implications of route, processing technique and timing of CMT administration could enable this comprehension. Such in vivo research should also incorporate an increased frequency of caecal swabbing following *C. jejuni* infection to gain a more consistent idea of infection dynamics within this replica-flock system. Any work in this regard should first determine the limit of detection for such techniques for improved accuracy. With products such as Aviguard, tested here, purporting maximal efficacy for prevention of colonisation from *Salmonella* species as oppose to *Campylobacter* species, extension of current protocols to a wider range of enteric pathogenic bacterial taxa might increase the scope of CMT protocols within the poultry industry. However, to maximise this potential, all work should consistently and accurately assess impacts of treatment on both feed consumption and total body weight.

With relatively limited information regarding the cell line utilised within the in vitro work described here, potential use of a widely described cell line (such as the macrophage HD11) might offer more viable results. To generate a more biologically relevant data set regarding immune response following CMT administration, and how this might subsequently impact *C. jejuni* colonisation, reliance on in vitro cell culture could also be superseded by use of gastrointestinal tissue sections collected from experimental birds. Performing RT-qPCR from RNA extracts of these tissues would enable complete characterisation of *C. jejuni* colonisation, microbiome composition and immune gene transcription for each individual experimental animal. Such information would prove invaluable in deciphering the complex biological mechanisms underpinning the phenotypic effects described within this primary research.

FINAL CONCLUSIONS

To conclude, the commercial broiler chicken represents an important infection reservoir for the zoonotic transfer of *Campylobacter* jejuni to global human populations. While posing an

evident risk to public health, the persistent intestinal, occasionally systemic, infection dynamics associated with *Campylobacter* in the broiler chicken are not as commensal as first believed and may also demonstrate important welfare indications for the chicken itself. The results presented here indicate that, at a time when effective *Campylobacter* control strategies are needed, early modulation of the chick microbiota may offer an effective means of protection. Although some-what rudimentary in concept, CMT shows promise in inducing rapid successional taxonomic changes in the chick microbiota to create an intestinal environment less susceptible to opportunistic infection. While it remains important to refine our understanding how CMT might influence both the intestinal microbiota and the immune system, such scientific advances bring us ever closer to development of an effective on-farm *Campylobacter* control strategy.

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Appendices

Appendix 1: Appendix to Chapter 2

ELISA for the detection of serum antibodies

Reagents:

- Carbonate-bicarbonate tables (Sigma, Poole, Dorset, UK)
- Nunc-immuno MicroWell flat-bottomed 96-well plates (sigma, Poole, Dorset, UK)
- Tween-20 sachets (Sigma, Poole, Dorset, UK)
- PBS
- Skimmed milk powder (Supermarket)
- Secondary antibody conjugated with alkaline phosphatase solution
- *P*-nitrophenyl phosphate (Sigma, Poole, Dorset, UK)
- NaOH tablets (FisherScientific, Loughborough, UK)

To do one day prior to assay

- Add 1 carbonate-bicarbonate tablet to 100ml deionised water
- Tale 10 ml carbonate-bicarbonate buffer (prepared above) and add soluble antigen to a concentration of 10 µg/ml
- Coat flat-bottomed 96-well plate with 100 µl antigen solution prepared and incubate overnight at 4°C

Assay protocol

- After overnight incubation, wash plates 3 x with a wash buffer (PBS + 0.05 % tween20; PBST)
- Incubate the plates with 200 µl blocking buffer for 1 hour at 37°C. Keep plate covered [blocking buffer: 3 % (w/v) skimmed milk powder in PBST]
- Wash plates once with wash buffer
- In a separate normal microplate, dilute blood serum samples in blocking buffer to required dilution
- Transfer 100 µl to each well of flat-bottomed plate in duplicate
- Incubate for 1 hour at 37°C. Keep plate covered
- Wash plates 3-5 times in wash buffer
- Add 100 µl of diluted secondary antibody conjugated with alkaline phosphatase solution to each well [Secondary antibody conjugated with alkaline phosphatase diluted with blocking solution, dilution will depend on antibody type]
- Incubate for 1 hour at 37°C.
- Wash 3-5 times in wash buffer
- Add 100 µl p-nitrophenyl phosphate to each well and incubate for 30 minutes at room temperature in the dark
- Add 100 µl 3N NaOH to each well to stop reaction (NaOH tablets dissolved in distilled water to desired concentration]
- Read absorbance at 405nm using microplate reader.

Figure 82. Laboratory protocol for laboratory ELISA analysis of chicken blood serum for IgA, IgM and IgY

Table 33. Bacteriological results corresponding to individual caecal content samples collected from *C. jejuni* infected experimental animals of experiment 1.

Group	Bird id	Sample weight	Dilution series	Bacterial colony count			CFU/ml	Log CFU/ml	Correction factor [1/sample weight]	CFU/g	Log CFU/g
				spot1	spot2	spot3					
Infected	**1934**	1.66	6	8	10	3	3.50E+08	8.54E+00	6.02E-01	2.11E+08	8.32E+00
Infected	**1935**	0.64	1	2	3	3.00	1.33E+03	3.12E+00	1.56E+00	2.08E+03	3.32E+00
Infected	**1936**	1.09	6	4	3	2.00	1.50E+08	8.18E+00	9.15E-01	1.37E+08	8.14E+00
Infected	**1937**	0.94	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1938**	1.15	4	3	2	1.00	1.00E+06	6.00E+00	8.73E-01	8.73E+05	5.94E+00
Infected	**1939**	2.23	6	8	3	3.00	2.33E+08	8.37E+00	4.48E-01	1.04E+08	8.02E+00
Infected	**1940**	1.50	6	7	7	1.00	2.50E+08	8.40E+00	6.66E-01	1.67E+08	8.22E+00
Infected	**1941**	1.92	5	6	3	4.00	2.17E+07	7.34E+00	5.21E-01	1.13E+07	7.05E+00
Infected	**1942**	1.01	5	7	2	5.00	2.33E+07	7.37E+00	9.95E-01	2.32E+07	7.37E+00
Infected	**1943**	0.80	4	11	7	8.00	4.33E+06	6.64E+00	1.24E+00	5.39E+06	6.73E+00
Infected	**1944**	3.10	4	9	13	13.00	5.83E+06	6.77E+00	3.23E-01	1.88E+06	6.27E+00
Infected	**1945**	0.86	6	3	6	6.00	2.50E+08	8.40E+00	1.16E+00	2.91E+08	8.46E+00
Infected	**1946**	0.76	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1947**	0.75	5	14	5	6.00	4.17E+07	7.62E+00	1.33E+00	5.53E+07	7.74E+00
Infected	**1948**	1.63	4	0	1	1.00	3.33E+05	5.52E+00	6.13E-01	2.04E+05	5.31E+00
Infected	**1949**	1.76	6	0	1	5	1.00E+08	8.00E+00	5.68E-01	5.68E+07	7.75E+00
Infected	**1950**	0.61	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1951**	1.38	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1952**	2.21	4	5	3	3	1.83E+06	6.26E+00	4.53E-01	8.31E+05	5.92E+00
Infected	**1953**	1.82	7	0	3	2	8.33E+08	8.92E+00	5.51E-01	4.59E+08	8.66E+00
Infected	**1954**	2.28	5	9	9	6	4.00E+07	7.60E+00	4.40E-01	1.76E+07	7.25E+00
Infected	**1955**	1.11	2	13	9	0	3.67E+04	4.56E+00	9.03E-01	3.31E+04	4.52E+00
Infected	**1956**	1.19	7	4	1	2	1.17E+09	9.07E+00	8.44E-01	9.85E+08	8.99E+00
Infected	**1957**	2.01	5	2	4	4	1.67E+07	7.22E+00	4.97E-01	8.28E+06	6.92E+00
Infected	**1958**	2.43	6	6	8	3	2.83E+08	8.45E+00	4.12E-01	1.17E+08	8.07E+00
Infected	**1959**	0.56	3	2	1	2	8.33E+04	4.92E+00	1.77E+00	1.48E+05	5.17E+00
Infected	**1962**	0.70	2	9	14	7	5.00E+04	4.70E+00	1.43E+00	7.15E+04	4.85E+00
Infected	**1963**	1.16	5	11	7	12.00	5.00E+07	7.70E+00	8.62E-01	4.31E+07	7.63E+00
Infected	**1964**	1.15	5	4	3	7.00	2.33E+07	7.37E+00	8.70E-01	2.03E+07	7.31E+00
Infected	**1965**	1.34	4	6	2	10.00	3.00E+06	6.48E+00	7.47E-01	2.24E+06	6.35E+00
Infected	**1966**	1.91	3	3	2	2.00	1.17E+05	5.07E+00	5.24E-01	6.11E+04	4.79E+00
Infected	**1967**	1.00	5	4	2	2	1.33E+07	7.12E+00	1.00E+00	1.34E+07	7.13E+00
Infected	**1968**	1.28	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1969**	1.77	7	19	16	16.00	8.50E+09	9.93E+00	**5.65E-01**	4.80E+09	9.68E+00
Infected	**1970**	0.94	4	5	5	8.00	3.00E+06	6.48E+00	1.07E+00	3.20E+06	6.50E+00
Infected	**1971**	0.59	5	13	14	12.00	6.50E+07	7.81E+00	1.69E+00	1.10E+08	8.04E+00
Infected	**1972**	2.36	4	12	8	17.00	6.17E+06	6.79E+00	4.24E-01	2.61E+06	6.42E+00
Infected	**1973**	0.79	6	7	12	11.00	5.00E+08	8.70E+00	1.26E+00	6.32E+08	8.80E+00
Infected	**1974**	1.11	6	13	7	8.00	4.67E+08	8.67E+00	8.99E-01	4.20E+08	8.62E+00
Infected	**1975**	1.24	5	1	1	2.00	6.67E+06	6.82E+00	8.05E-01	5.37E+06	6.73E+00
Infected	**1976**	2.75	4	9	13	8	5.00E+06	6.70E+00	3.64E-01	1.82E+06	6.26E+00
Infected	**1977**	0.63	3	1	1	3.00	8.33E+04	4.92E+00	1.59E+00	1.32E+05	5.12E+00
Infected	**1978**	2.19	6	16	15	13.00	7.33E+08	8.87E+00	4.56E-01	3.34E+08	8.52E+00
Infected	**1979**	1.84	7	1	1	1.00	5.00E+08	8.70E+00	5.43E-01	2.71E+08	8.43E+00
Infected	**1980**	1.65	5	9	2	6.00	2.83E+07	7.45E+00	6.06E-01	1.72E+07	7.23E+00
Infected	**1981**	0.89	4	9	6	4.00	3.17E+06	6.50E+00	1.12E+00	3.55E+06	6.55E+00
Infected	**1982**	0.75	4	6	13	8.00	4.50E+06	6.65E+00	1.33E+00	6.00E+06	6.78E+00
Infected	**1983**	1.59	4	11	8	9	4.67E+06	6.67E+00	6.31E-01	2.94E+06	6.47E+00
Infected	**1984**	1.29	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1985**	1.25	7	4	1	3.0	1.33E+09	9.12E+00	7.99E-01	1.07E+09	9.03E+00
Infected	**1986**	0.89	5	1	2	2.0	8.33E+06	6.92E+00	1.12E+00	9.36E+06	6.97E+00
Infected	**1987**	1.66	6	3	3	1	1.17E+08	8.07E+00	6.02E-01	7.03E+07	7.85E+00
Infected	**1988**	0.60	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1989**	1.20	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1990**	0.90	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1991**	0.46	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1992**	1.43	5	0	3	1	6.67E+06	6.82E+00	6.99E-01	4.66E+06	6.67E+00

Table 34. Bacteriological results corresponding to individual ileal content samples collected from *C. jejuni* infected experimental animals of experiment 1.

Group	Bird Id	Sample weight	Dilution series	Bacterial colony count			CFU/ml	log CFU/ml	Correction factor [1/sample weight]	CFU/g	log CFU/g
				spot1	spot2	spot3					
Infected	**1934**	1.47	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1935**	0.24	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1936**	0.35	3	5	5	9.00	3.17E+05	5.50E+00	2.83E+00	8.97E+05	5.95E+00
Infected	**1937**	0.47	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1938**	0.94	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1939**	0.68	4	2	2	4.00	1.33E+06	6.12E+00	1.47E+00	1.96E+06	6.29E+00
Infected	**1940**	1.38	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1941**	1.80	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1942**	0.56	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1943**	0.50	3	2	2	2.00	1.00E+05	5.00E+00	2.01E+00	2.01E+05	5.30E+00
Infected	**1944**	1.06	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1945**	0.75	2	10	0	0.00	1.67E+04	4.22E+00	1.33E+00	2.22E+04	4.35E+00
Infected	**1946**	0.43	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1947**	0.85	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1948**	1.09	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1949**	1.79	1	2	1	1	6.67E+02	2.82E+00	5.59E-01	3.72E+02	2.57E+00
Infected	**1950**	0.64	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1951**	0.54	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1952**	0.76	2	3	4	2	1.50E+04	4.18E+00	1.31E+00	1.96E+04	4.29E+00
Infected	**1953**	1.93	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1954**	2.19	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1955**	1.20	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1956**	1.73	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1957**	1.74	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1958**	0.71	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1959**	1.24	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1962**	1.72	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1963**	1.17	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1964**	1.10	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1965**	0.36	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1966**	1.23	1	0	2	3.00	8.33E+02	2.92E+00	8.13E-01	6.78E+02	2.83E+00
Infected	**1967**	1.23	5	0	2	2	6.67E+06	6.82E+00	8.14E-01	5.43E+06	6.73E+00
Infected	**1968**	0.58	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1969**	1.19	3	5	8	4	2.83E+05	5.45E+00	8.41E-01	2.38E+05	5.38E+00
Infected	**1970**	0.58	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1971**	0.48	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1972**	1.18	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1973**	0.71	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1974**	0.61	2	2	3	2.00	1.17E+04	4.07E+00	1.64E+00	1.92E+04	4.28E+00
Infected	**1975**	2.02	2	0	1	0.00	1.67E+03	3.22E+00	4.95E-01	8.24E+02	2.92E+00
Infected	**1976**	0.93	2	2	4	3	1.50E+04	4.18E+00	1.07E+00	1.61E+04	4.21E+00
Infected	**1977**	1.19	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1978**	1.55	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1979**	1.24	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1980**	1.03	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1981**	1.43	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1982**	0.53	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1983**	1.34	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1984**	1.07	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1985**	1.30	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1986**	0.99	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1987**	0.85	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1988**	0.74	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1989**	0.65	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1990**	0.29	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1991**	0.48	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**1992**	1.46	-	0	0	0	N/A	N/A	-	N/A	N/A

Table 35. Bacteriological results corresponding to individual caecal content samples collected from *C. jejuni* infected experimental animals of experiment 2.

Group	Bird Id	Sample weight	Dilution series	Bacterial colony count			CFU/ml	log CFU/ml	Correction factor [1/sample weight]	CFU/g	log CFU/g
				spot1	spot2	spot3					
Infected	**1**	2.60	4	4	4	2	1.67E+06	6.22E+00	3.85E-01	6.41E+05	5.81E+00
Infected	**2**	1.51	3	5	5	2	2.00E+05	5.30E+00	6.61E-01	1.32E+05	5.12E+00
Infected	**3**	0.78	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**4**	2.59	5	2	3	3	1.33E+07	7.12E+00	3.87E-01	5.15E+06	6.71E+00
Infected	**5**	1.76	3	6	4	5	2.50E+05	5.40E+00	5.67E-01	1.42E+05	5.15E+00
Infected	**6**	2.59	6	9	7	7	3.83E+08	8.58E+00	3.86E-01	1.48E+08	8.17E+00
Infected	**7**	1.92	6	3	9	8	3.33E+08	8.52E+00	5.21E-01	1.74E+08	8.24E+00
Infected	**8**	2.10	7	2	5	3	1.67E+09	9.22E+00	4.76E-01	7.94E+08	8.90E+00
Infected	**9**	1.44	5	17	6	3	4.33E+07	7.64E+00	6.94E-01	3.01E+07	7.48E+00
Infected	**10**	1.61	5	6	6	0	2.00E+07	7.30E+00	6.20E-01	1.24E+07	7.09E+00
Infected	**11**	2.31	6	1	11	10	3.67E+08	8.56E+00	4.33E-01	1.59E+08	8.20E+00
Infected	**12**	1.56	6	3	0	2	8.33E+07	7.92E+00	6.39E-01	5.33E+07	7.73E+00
Infected	**13**	1.66	1	2	4	3	1.50E+03	3.18E+00	6.01E-01	9.01E+02	2.95E+00
Infected	**14**	1.32	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**15**	1.18	3	3	2	1	1.00E+05	5.00E+00	8.49E-01	8.49E+04	4.93E+00
Infected	**16**	1.37	3	5	6	2	2.17E+05	5.34E+00	7.28E-01	1.58E+05	5.20E+00
Infected	**17**	0.66	5	0	0	6	1.00E+07	7.00E+00	1.51E+00	1.51E+07	7.18E+00
Infected	**18**	1.11	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**19**	1.99	5	6	3	8	2.83E+07	7.45E+00	5.03E-01	1.42E+07	7.15E+00
Infected	**20**	2.83	6	4	4	8	2.67E+08	8.43E+00	3.54E-01	9.43E+07	7.97E+00
Infected	**21**	1.86	6	6	5	5	2.67E+08	8.43E+00	5.38E-01	1.44E+08	8.16E+00
Infected	**22**	1.48	5	11	7	8	4.33E+07	7.64E+00	6.76E-01	2.93E+07	7.47E+00
Infected	**23**	1.77	5	7	14	9	5.00E+07	7.70E+00	5.65E-01	2.83E+07	7.45E+00
Infected	**24**	2.11	3	3	4	5	2.00E+05	5.30E+00	4.74E-01	9.48E+04	4.98E+00
Infected	**25**	2.61	4	7	12	10	4.83E+06	6.68E+00	3.83E-01	1.85E+06	6.27E+00
Infected	**26**	1.87	5	7	1	1	1.50E+07	7.18E+00	5.35E-01	8.03E+06	6.90E+00

Table 36. Bacteriological results corresponding to individual ileal content samples collected from *C. jejuni* infected experimental animals of experiment 2.

Group	Bird Id	Sample weight	Dilution series	Bacterial colony count			CFU/ml	log CFU/ml	Correction factor [1/sample weight]	CFU/g	log CFU/g
				spot1	spot2	spot3					
Infected	**1**	0.8	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**2**	1.203	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**3**	0.939	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**4**	0.668	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**5**	1.177	1	0	2	0	3.33E+03	3.52E+00	8.50E-01	2.83E+03	3.45E+00
Infected	**6**	0.726	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**7**	1.81	1	12	10	9	5.17E+04	4.71E+00	5.52E-01	2.85E+04	4.46E+00
Infected	**8**	0.722	2	6	6	7	3.17E+05	5.50E+00	1.39E+00	4.39E+05	5.64E+00
Infected	**9**	1.02	2	2	1	1	6.67E+04	4.82E+00	9.80E-01	6.54E+04	4.82E+00
Infected	**10**	1.961	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**11**	1.3	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**12**	1.655	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**13**	0.35	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**14**	0.988	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**15**	1.722	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**16**	0.397	2	0	0	1	1.67E+04	4.22E+00	2.52E+00	4.20E+04	4.62E+00
Infected	**17**	0.615	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**18**	1.77	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**19**	1.597	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**20**	0.486	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**21**	0.346	1	8	7	15	5.00E+04	4.70E+00	2.89E+00	1.45E+05	5.16E+00
Infected	**22**	1.047	1	4	3	3	1.67E+04	4.22E+00	9.55E-01	1.59E+04	4.20E+00
Infected	**23**	0.814	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**24**	2.181	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**25**	0.649	-	0	0	0	N/A	N/A	-	N/A	N/A
Infected	**26**	0.643	-	0	0	0	N/A	N/A	-	N/A	N/A

Appendix 2: Appendix to Chapter 3

Table 37. MALDI-TOF MS identifications from single colonies grown on CAB agar. Table shows organism of best and second-best match according to Score Value.

Condition	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
Aerobic	A1	*Bacillus pumilus*	1.82	*No Organism Identification Possible*	1.59
	A2	*Escherichia coli*	2.46	*Escherichia coli*	2.35
	A3b	*Escherichia coli*	2.45	*Escherichia coli*	2.33
	A4	*Enterococcus faecium*	2.31	*Enterococcus faecium*	2.22
	A5	*Bacillus pumilus*	1.99	*Bacillus pumilus*	1.83
	A6	*Bacillus altitudinis*	1.89	*Bacillus altitudinis*	1.84
	A7a	*Bacillus sp*	1.91	*Bacillus altitudinis*	1.88
	A7b	*Enterococcus faecalis*	2.30	*Enterococcus faecalis*	2.29
	A8a	*Bacillus cereus*	2.31	*Bacillus cereus*	2.18
	A8b	*Escherichia coli*	2.48	*Escherichia coli*	2.47
	A9a	*Bacillus subtilis*	N/A		
	A10a	*Solibacillus silvestris*	N/A		
	A10b	*Lactobacillus Paracesei*	1.78	*Staphylococcus cohnii*	1.73
Microaerobic	M1a	*Bacillus subtilis*	1.98	*Bacillus subtilis*	1.89
	M1a	*Bacillus mojavensis*	1.99	*Bacillus subtilis*	1.86
	M1a	*Bacillus amyloliquefaciens*	1.88	*Bacillus amyloliquefaciens*	1.84
	M1b	*Bacillus subtilis*	1.98	*Bacillus amyloliquefaciens_ssp_plantarum*	2.06
	M2a	*Bacillus subtilis*	1.96	*Bacillus subtilis*	1.91
	M2c	*Escherichia coli*	2.42	*Escherichia coli*	2.41
	M3	*Escherichia coli*	2.37	*Escherichia coli*	2.33
	M4a	*Enterococcus faecium*	2.08	*Enterococcus faecium*	1.98
	M4b	*Clostridium perfringens*	2.08	*Clostridium perfringens*	2.04
	M5	*Enterococcus faecium*	2.43	*Enterococcus faecium*	2.38
	M6a	*Bacillus subtilis*	1.94	*Bacillus subtilis*	1.84
	M6b	*Escherichia coli*	2.56	*Escherichia coli*	2.43
	M7	*Bacillus cereus*	2.42	*Bacillus cereus*	2.22
	M8	*Bacillus pumilus*	1.77	*Bacillus pumilus*	1.74
	M9a	*Bacillus megaterium*	2.22	*Bacillus megaterium*	1.98
	M9b	*Clostridium perfringens*	2.53	*Clostridium perfringens*	2.52
	M10a	*Bacillus megaterium*	2.02	*Bacillus megaterium*	1.92
	M10b	*Bacillus megaterium*	2.09	*Bacillus megaterium*	1.95
	M11	*Bacillus pumilus*	1.92	*Bacillus pumilus*	1.83
Anaerobic	An1	*Clostridium perfringens*	2.47	*Clostridium perfringens*	2.44
	An2	*Clostridium perfringens*	2.57	*Clostridium perfringens*	2.52
	An3	*Enterococcus faecium*	2.3	*Enterococcus faecium*	2.22

Table 38. Bacteriological results corresponding to individual caecal content samples collected from *C. jejuni* infected experimental animals of experiment 3.

Group	Bird ID	Sample weight	Dilution	spot1	spot2	spot3	cfu/ml	log cfu/ml	Correction factor	CFU/g	Log CFU/g
CMT	**949**	0.62	5	12	10	8	5.00E+08	8.70E+00	1.61E+00	8.06E+08	8.91E+00
CMT	**950**	1.53	1	4	5	5	2.33E+04	4.37E+00	6.54E-01	1.53E+04	4.18E+00
CMT	**951**	0.86	2	6	3	6	2.50E+05	5.40E+00	1.16E+00	2.90E+05	5.46E+00
CMT	**954**	0.36	1	3	1	1	8.33E+03	3.92E+00	2.80E+00	2.33E+04	4.37E+00
CMT	**955**	1.30	1	7	5	8	3.33E+04	4.52E+00	7.72E-01	2.57E+04	4.41E+00
CMT	**956**	0.67	2	14	11	16	6.83E+05	5.83E+00	1.50E+00	1.03E+06	6.01E+00
CMT	**958**	0.46	1	6	3	3	2.00E+04	4.30E+00	2.19E+00	4.39E+04	4.64E+00
CMT	**959**	0.50	1	10	12	11	5.50E+04	4.74E+00	1.98E+00	1.09E+05	5.04E+00
CMT	**961**	1.26	5	14	14	16	7.33E+08	8.87E+00	7.95E-01	5.83E+08	8.77E+00
CMT	**963**	0.79	1	1	2	2	8.33E+03	3.92E+00	1.26E+00	1.05E+04	4.02E+00
CMT	**966**	1.38	5	10	9	9	4.67E+08	8.67E+00	7.24E-01	3.38E+08	8.53E+00
CMT	**969**	0.54	1	4	2	6	2.00E+04	4.30E+00	1.84E+00	3.68E+04	4.57E+00
CMT	**970**	0.56	6	1	2	2	8.33E+08	8.92E+00	1.78E+00	1.48E+09	9.17E+00
CMT	**971**	0.45	1	1	3	2	1.00E+04	4.00E+00	2.21E+00	2.21E+04	4.34E+00
CMT	**972**	0.60	4	3	8	10	3.50E+07	7.54E+00	1.66E+00	5.81E+07	7.76E+00
CMT	**973**	0.83	5	13	16	16	7.50E+08	8.88E+00	1.20E+00	9.04E+08	8.96E+00
CMT	**974**	0.36	5	7	5	5	2.83E+08	8.45E+00	2.75E+00	7.78E+08	8.89E+00
CMT	**975**	0.53	3	5	4	2	1.83E+06	6.26E+00	1.88E+00	3.45E+06	6.54E+00
CMT	**925**	0.55	1	0	0	0	0.00E+00	N/A	1.81E+00	0.00E+00	N/A
Ext. Control	**1551**	0.79	8	18	23	22	1.05E+12	1.20E+01	1.27E+00	1.33E+12	1.21E+01
Ext. Control	**1552**	1.90	8	25	28	22	1.25E+12	1.21E+01	5.25E-01	6.57E+11	1.18E+01
Ext. Control	**1553**	0.64	8	18	17	23	9.67E+11	1.20E+01	1.55E+00	1.50E+12	1.22E+01
Ext. Control	**1554**	1.34	7	4	5	5	2.33E+10	1.04E+01	7.46E-01	1.74E+10	1.02E+01
Ext. Control	**1555**	0.19	8	22	25	27	1.23E+12	1.21E+01	5.15E+00	6.36E+12	1.28E+01
Ext. Control	**1556**	0.91	8	21	26	27	1.23E+12	1.21E+01	1.10E+00	1.35E+12	1.21E+01
Ext. Control	**1557**	1.25	8	10	18	15	7.17E+11	1.19E+01	8.01E-01	5.74E+11	1.18E+01
Ext. Control	**1559**	0.65	7	4	5	6	2.50E+10	1.04E+01	1.55E+00	3.87E+10	1.06E+01
Ext. Control	**1560**	0.41	8	31	28	29	1.47E+12	1.22E+01	2.46E+00	3.60E+12	1.26E+01
Ext. Control	**1561**	0.60	8	25	23	21	1.15E+12	1.21E+01	1.68E+00	1.93E+12	1.23E+01
Ext. Control	**1562**	0.77	8	25	25	29	1.32E+12	1.21E+01	1.29E+00	1.70E+12	1.22E+01
Ext. Control	**1563**	0.24	8	22	23	25	1.17E+12	1.21E+01	4.26E+00	4.96E+12	1.27E+01
Ext. Control	**1566**	0.33	8	16	14	13	7.17E+11	1.19E+01	3.01E+00	2.16E+12	1.23E+01
Ext. Control	**1567**	0.55	8	16	20	20	9.33E+11	1.20E+01	1.83E+00	1.71E+12	1.22E+01
Ext. Control	**1569**	0.58	8	23	25	28	1.27E+12	1.21E+01	1.71E+00	2.17E+12	1.23E+01
Ext. Control	**1570**	1.08	8	14	18	18	8.33E+11	1.19E+01	9.24E-01	7.70E+11	1.19E+01
Ext. Control	**1571**	0.49	8	17	16	15	8.00E+11	1.19E+01	2.06E+00	1.65E+12	1.22E+01
Ext. Control	**1572**	0.93	8	22	24	21	1.12E+12	1.20E+01	1.08E+00	1.20E+12	1.21E+01
Ext. Control	**1575**	0.42	8	12	9	7	4.67E+11	1.17E+01	2.38E+00	1.11E+12	1.20E+01

Table 39. Bacteriological results corresponding to individual ileal content samples collected from *C. jejuni* infected experimental animals of experiment 3.

Group	Bird ID	Sample weight	Dilution	spot1	spot2	spot3	cfu/ml	log cfu/ml	Correction factor	CFU/g	Log CFU/g
CMT	**949**	0.59	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**950**	0.42	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**951**	0.35	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**954**	0.43	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**955**	0.82	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**956**	0.37	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**958**	1.26	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**959**	0.80	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**961**	0.61	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**963**	0.35	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**966**	0.55	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**969**	0.62	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**970**	0.06	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**971**	0.34	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**972**	0.46	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**973**	0.56	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**974**	0.25	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**975**	0.40	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	**925**	0.48	-	0	0	0	N/A	N/A	-	N/A	N/A
Ext. Control	**1551**	0.82	-	0	0	0	N/A	N/A	-	N/A	N/A
Ext. Control	**1552**	0.71	1	2	2	1	8.33E+03	3.92E+00	1.41E+00	1.18E+04	4.07E+00
Ext. Control	**1553**	0.65	1	2	2	3	1.17E+04	4.07E+00	1.54E+00	1.80E+04	4.25E+00
Ext. Control	**1554**	1.15	1	1	2	1	6.67E+03	3.82E+00	8.68E-01	5.79E+03	3.76E+00
Ext. Control	**1555**	0.21	1	5	4	6	2.50E+04	4.40E+00	4.83E+00	1.21E+05	5.08E+00
Ext. Control	**1556**	0.41	-	0	0	0	N/A	N/A	-	N/A	N/A
Ext. Control	**1557**	0.50	1	8	2	5	2.50E+04	4.40E+00	2.00E+00	5.01E+04	4.70E+00
Ext. Control	**1559**	0.45	1	2	1	1	6.67E+03	3.82E+00	2.21E+00	1.47E+04	4.17E+00
Ext. Control	**1560**	0.74	2	4	2	2	1.33E+05	5.12E+00	1.35E+00	1.80E+05	5.26E+00
Ext. Control	**1561**	0.79	-	0	0	0	N/A	N/A	-	N/A	N/A
Ext. Control	**1562**	1.38	1	3	2	2	1.17E+04	4.07E+00	7.26E-01	8.47E+03	3.93E+00
Ext. Control	**1563**	0.65	3	10	12	13	5.83E+06	6.77E+00	1.54E+00	8.96E+06	6.95E+00
Ext. Control	**1566**	0.69	2	8	7	8	3.83E+05	5.58E+00	1.45E+00	5.57E+05	5.75E+00
Ext. Control	**1567**	0.31	1	4	3	2	1.50E+04	4.18E+00	3.18E+00	4.78E+04	4.68E+00
Ext. Control	**1569**	0.56	4	4	2	4	1.67E+07	7.22E+00	**1.78E+00**	2.97E+07	7.47E+00
Ext. Control	**1570**	0.43	1	4	5	5	2.33E+04	4.37E+00	2.33E+00	5.43E+04	4.73E+00
Ext. Control	**1571**	0.62	-	0	0	0	N/A	N/A	-	N/A	N/A
Ext. Control	**1572**	0.55	1	5	5	5	2.50E+04	4.40E+00	1.83E+00	4.59E+04	4.66E+00
Ext. Control	**1575**	0.53	2	3	3	3	1.50E+05	5.18E+00	1.87E+00	2.81E+05	5.45E+00

Table 40. Bacteriological results corresponding to individual caecal content samples collected from *C. jejuni* infected experimental animals of experiment 4.

Group	Bird Id	Sample weight	Dilution	Bacterial colony count			cfu/ml	log cfu/ml	Correction factor [1/sample weight	cfu/g	log cfu/g
				spot1	spot2	spot3					
CMT	**1801**	**1.964**	-	-	-	-	-	-	0.51	-	-
CMT	**1802**	**1.156**	-	-	-	-	-	-	0.87	-	-
CMT	**1803**	**1.593**	-	-	-	-	-	-	0.63	-	-
CMT	**1804**	**1.053**	-	-	-	-	-	-	0.95	-	-
CMT	**1805**	**1.871**	-	-	-	-	-	-	0.53	-	-
CMT	**1806**	**0.575**	-	-	-	-	-	-	1.74	-	-
CMT	**1808**	**1.51**	-	-	-	-	-	-	0.66	-	-
CMT	**1809**	**0.81**	-	-	-	-	-	-	1.23	-	-
Ext. Control	**3751**	**0.892**	6	9	5	10	4.00E+09	9.60E+00	1.12	4.48E+09	9.65E+00
Ext. Control	**3752**	**1.143**	3	2	4	6	2.00E+06	6.30E+00	0.87	1.75E+06	6.24E+00
Ext. Control	**3753**	**1.559**	5	7	9	16	5.33E+08	8.73E+00	0.64	3.42E+08	8.53E+00
Ext. Control	**3754**	**1.031**	4	8	10	5	3.83E+07	7.58E+00	0.97	3.72E+07	7.57E+00
Ext. Control	**3755**	**1.112**	5	6	4	2	2.00E+08	8.30E+00	0.90	1.80E+08	8.25E+00
Ext. Control	**3756**	**0.935**	6	4	3	4	1.83E+09	9.26E+00	1.07	1.96E+09	9.29E+00
Ext. Control	**3757**	**1.917**	5	12	19	22	8.83E+08	8.95E+00	0.52	4.61E+08	8.66E+00
Ext. Control	**3758**	**0.677**	6	5	7	8	3.33E+09	9.52E+00	1.48	4.92E+09	9.69E+00
Ext. Control	**3759**	**1.021**	5	19	11	16	7.67E+08	8.88E+00	0.98	7.51E+08	8.88E+00
Ext. Control	**3760**	**1.555**	5	9	7	12	4.67E+08	8.67E+00	0.64	3.00E+08	8.48E+00
Ext. Control	**3761**	**0.563**	5	3	6	5	2.33E+08	8.37E+00	1.78	4.14E+08	8.62E+00
Ext. Control	**3762**	**0.634**	4	11	6	9	4.33E+07	7.64E+00	1.58	6.83E+07	7.83E+00

Table 41. Bacteriological results corresponding to individual ileal content samples collected from *C. jejuni* infected experimental animals of experiment 4.

Group	Bird Id	Sample weight	Dilution	Bacterial colony count			cfu/ml	log cfu/ml	Correction factor [1/sample weight	cfu/g	log cfu/g
				spot1	spot2	spot3					
CMT	1801	0.41	-	-	-	-	-	-	2.44	-	-
CMT	1802	1.167	-	-	-	-	-	-	0.86	-	-
CMT	1803	0.794	-	-	-	-	-	-	1.26	-	-
CMT	1804	0.878	-	-	-	-	-	-	1.14	-	-
CMT	1805	0.598	-	-	-	-	-	-	1.67	-	-
CMT	1806	0.303	-	-	-	-	-	-	3.30	-	-
CMT	1808	0.807	-	-	-	-	-	-	1.24	-	-
CMT	1809	0.816	-	-	-	-	-	-	1.23	-	-
Ext. Control	3751	0.377	-	-	-	-	-	-	2.65	-	-
Ext. Control	3752	1.115	-	-	-	-	-	-	0.90	-	-
Ext. Control	3753	0.979	-	-	-	-	-	-	1.02	-	-
Ext. Control	3754	0.52	1	0	1	1	3.33E+03	3.52E+00	1.92	6.41E+03	3.81E+00
Ext. Control	3755	0.725	-	-	-	-	-	-	1.38	-	-
Ext. Control	3756	0.587	-	-	-	-	-	-	1.70	-	-
Ext. Control	3757	0.811	1	3	1	8	2.00E+04	4.30E+00	1.23	2.47E+04	4.39E+00
Ext. Control	3758	0.688	2	7	1	7	2.50E+05	5.40E+00	1.45	3.63E+05	5.56E+00
Ext. Control	3759	1.026	-	-	-	-	-	-	0.97	-	-
Ext. Control	3760	1.708	-	-	-	-	-	-	0.59	-	-
Ext. Control	3761	1.244	1	1	1	1	5.00E+03	3.70E+00	0.80	4.02E+03	3.60E+00
Ext. Control	3762	0.621	1	0	1	1	3.33E+03	3.52E+00	1.61	5.37E+03	3.73E+00

Table 42. Bacteriological results corresponding to individual caecal content samples collected from *C. jejuni* infected experimental animals of experiment 5.

Group	Bird Id	Sample weight	Dilution	Bacterial colony count			cfu/ml	log cfu/ml	Correction factor [1/sample weight	cfu/g	log cfu/g
				spot1	spot2	spot3					
CMT	**2251**	**1.074**	6	1	1	3	8.33E+08	8.92E+00	0.93	7.76E+08	8.89E+00
CMT	**2252**	**1.588**	6	13	11	9	5.50E+09	9.74E+00	0.63	3.46E+09	9.54E+00
CMT	**2253**	**0.694**	7	8	4	5	2.83E+10	1.05E+01	1.44	4.08E+10	1.06E+01
CMT	**2254**	**1.871**	4	3	2	4	1.50E+07	7.18E+00	0.53	8.02E+06	6.90E+00
CMT	**2255**	**1.92**	5	7	6	10	3.83E+08	8.58E+00	0.52	2.00E+08	8.30E+00
CMT	**2256**	**1.662**	5	7	9	11	4.50E+08	8.65E+00	0.60	2.71E+08	8.43E+00
CMT	**2257**	**1.335**	4	6	6	5	2.83E+07	7.45E+00	0.75	2.12E+07	7.33E+00
CMT	**2258**	**0.875**	6	7	6	7	3.33E+09	9.52E+00	1.14	3.81E+09	9.58E+00
CMT	**2259**	**0.808**	5	2	5	5	2.00E+08	8.30E+00	1.24	2.48E+08	8.39E+00
CMT	**2260**	**0.691**	5	7	4	8	3.17E+08	8.50E+00	1.45	4.58E+08	8.66E+00
CMT	**2261**	**1.712**	5	1	1	3	8.33E+07	7.92E+00	0.58	4.87E+07	7.69E+00
CMT	**2262**	**2.118**	5	7	8	9	4.00E+08	8.60E+00	0.47	1.89E+08	8.28E+00
CMT	**2263**	**1.07**	6	25	21	28	1.23E+10	1.01E+01	0.93	1.15E+10	1.01E+01
CMT	**2264**	**1.661**	5	11	17	13	6.83E+08	8.83E+00	0.60	4.11E+08	8.61E+00
CMT	**2265**	**1.387**	5	3	2	3	1.33E+08	8.12E+00	0.72	9.61E+07	7.98E+00
Ext. Control	**1776**	**1.272**	5	2	9	11	3.67E+08	8.56E+00	0.79	2.88E+08	8.46E+00
Ext. Control	**1777**	**1.342**	6	1	1	2	6.67E+08	8.82E+00	0.75	4.97E+08	8.70E+00
Ext. Control	**1778**	**2.632**	5	1	2	4	1.17E+08	8.07E+00	0.38	4.43E+07	7.65E+00
Ext. Control	**1779**	**1.129**	3	7	5	5	2.83E+06	6.45E+00	0.89	2.51E+06	6.40E+00
Ext. Control	**1780**	**2.753**	4	2	2	3	1.17E+07	7.07E+00	0.36	4.24E+06	6.63E+00
Ext. Control	**1781**	**1.264**	6	7	7	4	3.00E+09	9.48E+00	0.79	2.37E+09	9.38E+00
Ext. Control	**1782**	**0.822**	3	9	10	14	5.50E+06	6.74E+00	1.22	6.69E+06	6.83E+00
Ext. Control	**1783**	**1.497**	6	3	1	1	8.33E+08	8.92E+00	0.67	5.57E+08	8.75E+00
Ext. Control	**1784**	**1.364**	4	8	12	9	4.83E+07	7.68E+00	0.73	3.54E+07	7.55E+00
Ext. Control	**1785**	**1.496**	5	4	8	9	3.50E+08	8.54E+00	0.67	2.34E+08	8.37E+00
Ext. Control	**1786**	**0.637**	4	3	3	0	1.00E+07	7.00E+00	1.57	1.57E+07	7.20E+00
Ext. Control	**1788**	**0.895**	5	3	3	1	1.17E+08	8.07E+00	1.12	1.30E+08	8.12E+00
Ext. Control	**1789**	**0.151**	6	2	9	6	2.83E+09	9.45E+00	6.62	1.88E+10	1.03E+01
Ext. Control	**1790**	**1.949**	3	30	28	37	1.58E+07	7.20E+00	0.51	8.12E+06	6.91E+00
Ext. Control	**1791**	**1.724**	5	4	10	7	3.50E+08	8.54E+00	0.58	2.03E+08	8.31E+00
Ext. Control	**1792**	**1.575**	5	10	7	5	3.67E+08	8.56E+00	0.63	2.33E+08	8.37E+00

Table 43. Bacteriological results corresponding to individual ileal content samples collected from *C. jejuni* infected experimental animals of experiment 5.

Sample	Bird Id	Sample weight	Dilution	Bacterial colony count			cfu/ml	log cfu/ml	Correction factor [1/sample weight	cfu/g	log cfu/g
				spot1	spot2	spot3					
Ileum	**2251**	**1.219**	-	-	-	-	-	-	0.82	-	
Ileum	**2252**	**0.582**	-	-	-	-	-	-	1.72	-	
Ileum	**2253**	**1.546**	1	9	9	3	3.50E+04	4.54E+00	0.65	2.26E+04	4.35E+00
Ileum	**2254**	**1.194**	-	-	-	-	-	-	0.84	-	
Ileum	**2255**	**1.521**	-	-	-	-	-	-	0.66	-	
Ileum	**2256**	**1.854**	1	2	7	6	2.50E+04	4.40E+00	0.54	1.35E+04	4.13E+00
Ileum	**2257**	**1.358**	1	10	13	7	5.00E+04	4.70E+00	0.74	3.68E+04	4.57E+00
Ileum	**2258**	**1.082**	1	41	40	36	1.95E+05	5.29E+00	0.92	1.80E+05	5.26E+00
Ileum	**2259**	**0.934**	-	-	-	-	-	-	1.07	-	
Ileum	**2260**	**0.249**	-	-	-	-	-	-	4.02	-	
Ileum	**2261**	**0.986**	-	-	-	-	-	-	1.01	-	
Ileum	**2262**	**1.891**	-	-	-	-	-	-	0.53	-	
Ileum	**2263**	**0.601**	-	-	-	-	-	-	1.66	-	
Ileum	**2264**	**0.664**	1	30	32	28	1.50E+05	5.18E+00	1.51	2.26E+05	5.35E+00
Ileum	**2265**	**0.942**	1	9	11	12	5.33E+04	4.73E+00	1.06	5.66E+04	4.75E+00
Ileum	**1776**	**0.998**	-	-	-	-	-	-	1.00	-	
Ileum	**1777**	**1.236**	1	60	70	65	3.25E+05	5.51E+00	0.81	2.63E+05	5.42E+00
Ileum	**1778**	**0.535**	-	-	-	-	-	-	1.87	-	
Ileum	**1779**	**0.869**	-	-	-	-	-	-	1.15	-	
Ileum	**1780**	**1.305**	1	70	66	63	3.32E+05	5.52E+00	0.77	2.54E+05	5.41E+00
Ileum	**1781**	**2.072**	1	52	48	62	2.70E+05	5.43E+00	0.48	1.30E+05	5.11E+00
Ileum	**1782**	**0.544**	-	-	-	-	-	-	1.84	-	
Ileum	**1783**	**1.361**	-	-	-	-	-	-	0.73	-	
Ileum	**1784**	**0.895**	-	-	-	-	-	-	1.12	-	
Ileum	**1785**	**2.098**	1	3	11	11	4.17E+04	4.62E+00	0.48	1.99E+04	4.30E+00
Ileum	**1786**	**0.679**	-	-	-	-	-	-	1.47	-	
Ileum	**1788**	**0.847**	-	-	-	-	-	-	1.18	-	
Ileum	**1789**	**0.763**	1	10	12	14	6.00E+04	4.78E+00	1.31	7.86E+04	4.90E+00
Ileum	**1790**	**0.797**	-	-	-	-	-	-	1.25	-	
Ileum	**1791**	**0.943**	-	-	-	-	-	-	1.06	-	
Ileum	**1792**	**1.073**	1	42	38	56	2.27E+05	5.36E+00	0.93	2.11E+05	5.32E+00

Table 44. Bacteriological results corresponding to individual caecal content samples collected from C. jejuni infected experimental animals of experiment 6.

	Group	Bird Id	Sample Weight	Dilution	Bacterial colony count			CFU/ml	log CFU/ml	Correction factor [1/sample weight	CFU/g	logCFU/g
					1	2	3					
4 d.p.i	CMT	1801	1.173	-	-	-	-	-	-	0.85	0.00E+00	NA
		1802	0.924	-	-	-	-	-	-	1.08	0.00E+00	NA
		1803	1.074	-	-	-	-	-	-	0.93	0.00E+00	NA
		1804	0.456	-	-	-	-	-	-	2.19	0.00E+00	NA
		1805	1.793	-	-	-	-	-	-	0.56	0.00E+00	NA
		1806	1.645	-	-	-	-	-	-	0.61	0.00E+00	NA
		1814	0.828	-	-	-	-	-	-	1.21	0.00E+00	NA
		1815	0.837	-	-	-	-	-	-	1.19	0.00E+00	NA
		1816	1.968	-	-	-	-	-	-	0.51	0.00E+00	NA
		1819	1.431	-	-	-	-	-	-	0.70	0.00E+00	NA
	Ext. Control	2151	0.571	-	-	-	-	-	-	1.75	0.00E+00	NA
		2156	2.206	-	-	-	-	-	-	0.45	0.00E+00	NA
		2157	2.257	-	-	-	-	-	-	0.44	0.00E+00	NA
		2164	2.468	-	-	-	-	-	-	0.41	0.00E+00	NA
		2168	1.682	-	-	-	-	-	-	0.59	0.00E+00	NA
		2176	0.778	1	0	2	0	3.33E+03	3.52E+00	1.29	4.28E+03	3.63E+00
		2179	1.828	1	2	2	1	8.33E+03	3.92E+00	0.55	4.56E+03	3.66E+00
		2181	2.679	-	-	-	-	-	-	0.37	0.00E+00	NA
		2183	0.508	-	-	-	-	-	-	1.97	0.00E+00	NA
		2184	0.734	-	-	-	-	-	-	1.36	0.00E+00	NA
		2189	0.73	1	1	2	3	1.00E+04	4.00E+00	1.37	1.37E+04	4.14E+00
		2191	0.8	-	-	-	-		-	1.25	0.00E+00	NA
10 d.p.i	CMT	1807	1.4	5	6	4	4	2.33E+08	8.37E+00	0.71	1.67E+08	8.22E+00
		1808	1.311	-	-	-	-	-	-	0.76	0.00E+00	NA
		1809	1.973	6	0	2	4	1.00E+09	9.00E+00	0.51	5.07E+08	8.70E+00
		1810	0.355	6	3	1	2	1.00E+09	9.00E+00	2.82	2.82E+09	9.45E+00
		1811	0.731	6	1	1	1	5.00E+08	8.70E+00	1.37	6.84E+08	8.84E+00
		1812	0.616	6	2	2	2	1.00E+09	9.00E+00	1.62	1.62E+09	9.21E+00
		1813	0.879	4	6	8	4	3.00E+07	7.48E+00	1.14	3.41E+07	7.53E+00
		1817	1.831	4	2	1	1	6.67E+06	6.82E+00	0.55	3.64E+06	6.56E+00
		1820	1.313	3	1	1	4	1.00E+06	6.00E+00	0.76	7.62E+05	5.88E+00
		1821	1.833	-	-	-	-	-	-	0.55	0.00E+00	NA
	Ext. Control	2161	1.103	2	3	3	1	1.17E+05	5.07E+00	0.91	1.06E+05	5.02E+00
		2162	0.571	5	4	3	2	1.50E+08	8.18E+00	1.75	2.63E+08	8.42E+00
		2163	1.228	5	1	2	1	6.67E+07	7.82E+00	0.81	5.43E+07	7.73E+00
		2165	1.905	6	3	2	4	1.50E+09	9.18E+00	0.52	7.87E+08	8.90E+00
		2166	1.558	5	5	6	4	2.50E+08	8.40E+00	0.64	1.60E+08	8.21E+00
		2167	0.938	5	11	13	14	6.33E+08	8.80E+00	1.07	6.75E+08	8.83E+00
		2170	2.005	5	2	9	9	3.33E+08	8.52E+00	0.50	1.66E+08	8.22E+00
		2172	1.171	-	-	-	-			0.85	0.00E+00	NA
		2178	1.34	4	1	4	3	1.33E+07	7.12E+00	0.75	9.95E+06	7.00E+00
		2180	0.776	6	5	1	3	1.50E+09	9.18E+00	1.29	1.93E+09	9.29E+00
		2182	0.908	6	5	6	5	2.67E+09	9.43E+00	1.10	2.94E+09	9.47E+00
		2193	1.54	4	3	2	1	1.00E+07	7.00E+00	0.65	6.49E+06	6.81E+00

Table 45. Bacteriological results corresponding to individual ileal content samples collected from *C. jejuni* infected experimental animals of experiment 6.

	Group	Bird Id	Sample Weight	Dilution	Bacterial colony count			CFU/ml	log CFU/ml	Correction factor [1/sample weight	CFU/g	logCFU/g
					spot1	spot2	spot3					
4 d.p.i	CMT	1801	0.689	-	-	-	-			1.45	0.00E+00	NA
	CMT	1802	0.855	-	-	-	-			1.17	0.00E+00	NA
	CMT	1803	0.645	-	-	-	-			1.55	0.00E+00	NA
	CMT	1804	0.569	-	-	-	-			1.76	0.00E+00	NA
	CMT	1805	1.345	-	-	-	-			0.74	0.00E+00	NA
	CMT	1806	0.718	-	-	-	-			1.39	0.00E+00	NA
	CMT	1814	0.572	-	-	-	-			1.75	0.00E+00	NA
	CMT	1815	0.97	-	-	-	-			1.03	0.00E+00	NA
	CMT	1816	0.244	-	-	-	-			4.10	0.00E+00	NA
	CMT	1819	0.783	-	-	-	-			1.28	0.00E+00	NA
	Ext. Control	2151	0.804	1	1	0	0	1.67E+03	3.22E+00	1.24	2.07E+03	3.32E+00
	Ext. Control	2156	1.017	-	-	-	-			0.98	0.00E+00	NA
	Ext. Control	2157	1.464	-	-	-	-			0.68	0.00E+00	NA
	Ext. Control	2164	1.199	1	2	2	0	6.67E+03	3.82E+00	0.83	5.56E+03	3.75E+00
	Ext. Control	2168	1.23	-	-	-	-			0.81	0.00E+00	NA
	Ext. Control	2176	0.709	-	-	-	-			1.41	0.00E+00	NA
	Ext. Control	2179	0.817	-	-	-	-			1.22	0.00E+00	NA
	Ext. Control	2181	1.007	-	-	-	-			0.99	0.00E+00	NA
	Ext. Control	2183	0.522	-	-	-	-			1.92	0.00E+00	NA
	Ext. Control	2184	1.287	-	-	-	-			0.78	0.00E+00	NA
	Ext. Control	2189	0.301	-	-	-	-			3.32	0.00E+00	NA
	Ext. Control	2191	0.642	-	-	-	-			1.56	0.00E+00	NA
10 d.p.i	CMT	1807	1.085	1	2	2	2	1.00E+04	4.00E+00	0.92	9.22E+03	3.96E+00
	CMT	1808	0.621	-	-	-	-			1.61	0.00E+00	NA
	CMT	1809	1.476	2	4	10	8	3.67E+05	5.56E+00	0.68	2.48E+05	5.40E+00
	CMT	1810	0.7	3	8	7	11	4.33E+06	6.64E+00	1.43	6.19E+06	6.79E+00
	CMT	1811	0.274	1	1	1	2	6.67E+03	3.82E+00	3.65	2.43E+04	4.39E+00
	CMT	1812	0.515	2	3	6	4	2.17E+05	5.34E+00	1.94	4.21E+05	5.62E+00
	CMT	1813	0.769	2	2	2	1	8.33E+04	4.92E+00	1.30	1.08E+05	5.03E+00
	CMT	1817	1.017	2	7	8	3	3.00E+05	5.48E+00	0.98	2.95E+05	5.47E+00
	CMT	1820	0.542	-	-	-	-			1.85	0.00E+00	NA
	CMT	1821	0.965	-	-	-	-			1.04	0.00E+00	NA
	Ext. Control	2161	1.079	-	-	-	-			0.93	0.00E+00	NA
	Ext. Control	2162	1.179	1	0	1	0	1.67E+03	3.22E+00	0.85	1.41E+03	3.15E+00
	Ext. Control	2163	0.553	-	-	-	-			1.81	0.00E+00	NA
	Ext. Control	2165	0.815	2	1	3	4	1.33E+05	5.12E+00	1.23	1.64E+05	5.21E+00
	Ext. Control	2166	1.17	1	4	3	4	1.83E+04	4.26E+00	0.85	1.57E+04	4.20E+00
	Ext. Control	2167	0.993	1	4	0	4	1.33E+04	4.12E+00	1.01	1.34E+04	4.13E+00
	Ext. Control	2170	0.932	1	0	2	0	3.33E+03	3.52E+00	1.07	3.58E+03	3.55E+00
	Ext. Control	2172	0.706	-	-	-	-			1.42	0.00E+00	NA
	Ext. Control	2178	0.947	1	11	7	9	4.50E+04	4.65E+00	1.06	4.75E+04	4.68E+00
	Ext. Control	2180	0.395	2	3	2	3	1.33E+05	5.12E+00	2.53	3.38E+05	5.53E+00
	Ext. Control	2182	0.987	1	4	8	5	2.83E+04	4.45E+00	1.01	2.87E+04	4.46E+00
	Ext. Control	2193	1.277	1	1	2	0	5.00E+03	3.70E+00	0.78	3.92E+03	3.59E+00

Appendix 3: Appendix to Chapter 4

Table 46. Bacteriological results corresponding to individual caecal content samples collected from *C. jejuni* infected experimental animals of experiment 7.

Group	Bird Id	Sample weight	Dilution	Bacterial Count			CFU/ml	log CFU/ml	Correction factor	CFU/g	Log CFU/g
				spot1	spot2	spot3					
CMT	1703	0.51	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1704	0.66	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1705	0.87	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1706	2.52	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1707	1.94	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1710	2.67	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1712	1.55	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1713	1.12	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1714	1.28	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1715	0.87	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1717	1.786	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1718*	2.54	1	0	1	0	1.67E+03	3.22E+00	3.94E-01	6.56E+02	2.82E+00
CMT	1719*	1.61	-	0	0	0	N/A	N/A	-	N/A	N/A
Aviguard	3651	3.04	5	3	2	4	1.50E+08	8.18E+00	3.29E-01	4.93E+07	7.69E+00
Aviguard	3652*	2.64	5	1	2	1	6.67E+07	7.82E+00	3.79E-01	2.53E+07	7.40E+00
Aviguard	3653	2.96	3	7	10	8	4.17E+06	6.62E+00	3.38E-01	1.41E+06	6.15E+00
Aviguard	3654*	2.05	5	1	3	2	1.00E+08	8.00E+00	4.88E-01	4.88E+07	7.69E+00
Aviguard	3655	2.25	6	3	2	2	1.17E+09	9.07E+00	4.44E-01	5.19E+08	8.71E+00
Aviguard	3657	2.26	5	8	3	6	2.83E+08	8.45E+00	4.42E-01	1.25E+08	8.10E+00
Aviguard	3659	0.79	5	2	1	3	1.00E+08	8.00E+00	1.27E+00	1.27E+08	8.10E+00
Aviguard	3660	1.13	5	3	4	9	2.67E+08	8.43E+00	8.85E-01	2.36E+08	8.37E+00
Aviguard	3661	1.64	6	2	3	2	1.17E+09	9.07E+00	6.10E-01	7.11E+08	8.85E+00
Aviguard	3662	1.16	5	2	4	4	1.67E+08	8.22E+00	8.62E-01	1.44E+08	8.16E+00
Aviguard	3663	1.155	5	1	1	0	3.33E+07	7.52E+00	8.66E-01	2.89E+07	7.46E+00
Aviguard	3664	2.74	5	1	1	2	6.67E+07	7.82E+00	3.65E-01	2.43E+07	7.39E+00
Aviguard	3665	1.99	6	2	4	3	1.50E+09	9.18E+00	5.03E-01	7.54E+08	8.88E+00
Int. Control	226	1.326	6	1	2	4	1.17E+09	9.07E+00	7.54E-01	8.80E+08	8.94E+00
Int. Control	227	2.161	5	2	4	6	2.00E+08	8.30E+00	4.63E-01	9.25E+07	7.97E+00
Int. Control	228	1.455	4	7	7	9	3.83E+07	7.58E+00	6.87E-01	2.63E+07	7.42E+00
Int. Control	229	0.363	6	8	5	6	3.17E+09	9.50E+00	2.75E+00	8.72E+09	9.94E+00
Int. Control	230	0.865	6	2	6	3	1.83E+09	9.26E+00	1.16E+00	2.12E+09	9.33E+00
Int. Control	231*	1.394	7	2	5	4	1.83E+10	1.03E+01	7.17E-01	1.32E+10	1.01E+01
Int. Control	232	1.619	6	4	1	4	1.50E+09	9.18E+00	6.18E-01	9.26E+08	8.97E+00
Int. Control	233*	1.215	5	6	3	3	2.00E+08	8.30E+00	8.23E-01	1.65E+08	8.22E+00
Int. Control	234	2.282	6	1	1	1	5.00E+08	8.70E+00	4.38E-01	2.19E+08	8.34E+00
Int. Control	236	1.359	5	12	7	12	5.17E+08	8.71E+00	7.36E-01	3.80E+08	8.58E+00
Int. Control	237	2.331	6	6	5	5	2.67E+09	9.43E+00	4.29E-01	1.14E+09	9.06E+00
Int. Control	238	2.647	6	1	2	1	6.67E+08	8.82E+00	3.78E-01	2.52E+08	8.40E+00
Int. Control	239	1.836	7	8	3	1	2.00E+10	1.03E+01	5.45E-01	1.09E+10	1.00E+01
Ext. Control	802*	1.94	4	8	5	6	3.17E+07	7.50E+00	5.15E-01	1.63E+07	7.21E+00
Ext. Control	803	2.11	5	4	8	9	3.50E+08	8.54E+00	4.74E-01	1.66E+08	8.22E+00
Ext. Control	804	2.71	6	5	7	4	2.67E+09	9.43E+00	3.69E-01	9.84E+08	8.99E+00
Ext. Control	805	2.068	5	9	8	11	4.67E+08	8.67E+00	4.84E-01	2.26E+08	8.35E+00
Ext. Control	806	1.692	5	4	4	4	2.00E+08	8.30E+00	5.91E-01	1.18E+08	8.07E+00
Ext. Control	807	1.58	2	13	9	6	4.67E+05	5.67E+00	6.33E-01	2.95E+05	5.47E+00
Ext. Control	809	1.07	4	13	15	16	7.33E+07	7.87E+00	9.35E-01	6.85E+07	7.84E+00
Ext. Control	810	1.92	5	14	12	8	5.67E+08	8.75E+00	5.21E-01	2.95E+08	8.47E+00
Ext. Control	811	1.04	5	3	5	2	1.67E+08	8.22E+00	9.62E-01	1.60E+08	8.20E+00
Ext. Control	812	1.71	-	0	0	0	N/A	N/A	-	N/A	N/A
Ext. Control	813	2.66	6	2	1	5	1.33E+09	9.12E+00	3.76E-01	5.01E+08	8.70E+00
Ext. Control	814*	0.885	3	2	4	5	1.83E+06	6.26E+00	1.13E+00	2.07E+06	6.32E+00
Ext. Control	815	2.58	3	2	2	1	8.33E+05	5.92E+00	3.88E-01	3.23E+05	5.51E+00

Table 47. Bacteriological results corresponding to individual ileal content samples collected from *C. jejuni* infected experimental animals of experiment 7.

Group	Bird Id	Sample weight	Dilution	Bacterial Count			CFU/ml	Log CFU/ml	Correction factor	CFU/g	Log CFU/g
				spot1	spot2	spot3					
CMT	1703	1.216	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1704	1.288	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1705	0.841	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1706	1.194	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1707	0.916	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1710	1.527	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1712	0.94	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1713	0.904	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1714	1.405	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1715	0.899	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1717	1.029	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1718*	0.996	-	0	0	0	N/A	N/A	-	N/A	N/A
CMT	1719*	0.805	-	0	0	0	N/A	N/A	-	N/A	N/A
Aviguard	3651	1.169	-	0	0	0	N/A	N/A	-	N/A	N/A
Aviguard	3652*	0.491	-	0	0	0	N/A	N/A	-	N/A	N/A
Aviguard	3653	0.788	1	2	1	1	6.67E+03	3.82E+00	1.27E+00	8.46E+03	3.93E+00
Aviguard	3654*	1.239	2	4	6	4	2.33E+05	5.37E+00	8.07E-01	1.88E+05	5.27E+00
Aviguard	3655	1.374	1	3	4	7	2.33E+04	4.37E+00	7.28E-01	1.70E+04	4.23E+00
Aviguard	3657	1.259	1	3	7	5	2.50E+04	4.40E+00	7.94E-01	1.99E+04	4.30E+00
Aviguard	3659	0.966	1	4	5	3	2.00E+04	4.30E+00	1.04E+00	2.07E+04	4.32E+00
Aviguard	3660	1.138	2	7	7	7	3.50E+05	5.54E+00	8.79E-01	3.08E+05	5.49E+00
Aviguard	3661	0.808	2	2	4	3	1.50E+05	5.18E+00	1.24E+00	1.86E+05	5.27E+00
Aviguard	3662	1.136	-	0	0	0	N/A	N/A	-	N/A	N/A
Aviguard	3663	0.519	1	1	0	2	5.00E+03	3.70E+00	1.93E+00	9.63E+03	3.98E+00
Aviguard	3664	0.701	2	1	3	2	1.00E+05	5.00E+00	1.43E+00	1.43E+05	5.15E+00
Aviguard	3665	1.881	3	1	1	1	5.00E+05	5.70E+00	5.32E-01	2.66E+05	5.42E+00
Int. Control	226	1.513	-	0	0	0	N/A	N/A	-	N/A	N/A
Int. Control	227	1.059	-	0	0	0	N/A	N/A	-	N/A	N/A
Int. Control	228	0.744	2	1	0	0	1.67E+04	4.22E+00	1.34E+00	2.24E+04	4.35E+00
Int. Control	229	0.796	5	12	9	14	5.83E+08	8.77E+00	1.26E+00	7.33E+08	8.87E+00
Int. Control	230	1.381	3	0	3	3	1.00E+06	6.00E+00	7.24E-01	7.24E+05	5.86E+00
Int. Control	231*	1.544	3	7	8	6	3.50E+06	6.54E+00	6.48E-01	2.27E+06	6.36E+00
Int. Control	232	1.131	-	0	0	0	N/A	N/A	-	N/A	N/A
Int. Control	233*	0.986	2	2	5	2	1.50E+05	5.18E+00	1.01E+00	1.52E+05	5.18E+00
Int. Control	234	0.886	-	0	0	0	N/A	N/A	-	N/A	N/A
Int. Control	236	1.253	2	2	4	2	1.33E+05	5.12E+00	7.98E-01	1.06E+05	5.03E+00
Int. Control	237	0.727	4	1	3	1	8.33E+06	6.92E+00	1.38E+00	1.15E+07	7.06E+00
Int. Control	238	1.03	1	1	0	0	1.67E+03	3.22E+00	9.71E-01	1.62E+03	3.21E+00
Int. Control	239	1.203	1	0	0	1	1.67E+03	3.22E+00	8.31E-01	1.39E+03	3.14E+00
Ext. Control	802*	1.962	1	3	5	4	2.00E+04	4.30E+00	5.10E-01	1.02E+04	4.01E+00
Ext. Control	803	1.476	1	5	8	7	3.33E+04	4.52E+00	6.78E-01	2.26E+04	4.35E+00
Ext. Control	804	0.661	1	2	0	0	3.33E+03	3.52E+00	1.51E+00	5.04E+03	3.70E+00
Ext. Control	805	0.748	1	3	1	1	8.33E+03	3.92E+00	1.34E+00	1.11E+04	4.05E+00
Ext. Control	806	0.954	1	1	2	4	1.17E+04	4.07E+00	1.05E+00	1.22E+04	4.09E+00
Ext. Control	807	1.356	-	0	0	0	N/A	N/A	-	N/A	N/A
Ext. Control	809	1.083	1	6	6	3	2.50E+04	4.40E+00	9.23E-01	2.31E+04	4.36E+00
Ext. Control	810	0.713	1	1	1	1	5.00E+03	3.70E+00	1.40E+00	7.01E+03	3.85E+00
Ext. Control	811	1.192	1	1	2	3	1.00E+04	4.00E+00	8.39E-01	8.39E+03	3.92E+00
Ext. Control	812	0.253	-	0	0	0	N/A	N/A	-	N/A	N/A
Ext. Control	813	1.782	-	0	0	0	N/A	N/A	-	N/A	N/A
Ext. Control	814*	1.425	-	0	0	0	N/A	N/A	-	N/A	N/A
Ext. Control	815	0.887	-	0	0	0	N/A	N/A	-	N/A	N/A

Table 48. Recovered *C. jejuni* M1 and percentage invasion following GPA protocols on 8E11 cell lines.

Invasion Repeat	Treatment	Replicate	Miles Misra dilution	Count 1	Count 2	Count 3	Average colony count	CFU/mL	Log10 CFU/mL	Invasion (%)
1	Inoculate count	N/A	4	27	30	29	28.67	1.43E+07	7.16	N/A
	MHB (Control)	C1	2	19	83	43	48.33	2.42E+05	5.38	1.69
		C2	2	22	32	37	30.33	1.52E+05	5.18	1.06
		C3	2	23	22	36	27.00	1.35E+05	5.13	0.94
		C4	2	18	12	35	21.67	1.08E+05	5.03	0.76
		C5	2	21	12	10	14.33	7.17E+04	4.86	0.50
		C6	2	10	18	9	12.33	6.17E+04	4.79	0.43
		C7	2	13	24	17	18.00	9.00E+04	4.95	0.63
		C8	3	19	21	27	22.33	1.12E+06	6.05	7.79
		C9	2	29	16	17	20.67	1.03E+05	5.01	0.72
		C10	2	13	8	9	10.00	5.00E+04	4.70	0.35
		C11	2	24	34	28	28.67	1.43E+05	5.16	1.00
		C12	2	63	35	15	37.67	1.88E+05	5.27	1.31
	CMT	FT1	2	10	18	10	12.67	6.33E+04	4.80	0.44
		FT2	2	14	10	16	13.33	6.67E+04	4.82	0.47
		FT3	2	8	7	11	8.67	4.33E+04	4.64	0.30
		FT4	2	13	13	18	14.67	7.33E+04	4.87	0.51
		FT5	2	7	12	13	10.67	5.33E+04	4.73	0.37
		FT6	2	16	16	12	14.67	7.33E+04	4.87	0.51
		FT7	2	13	13	19	15.00	7.50E+04	4.88	0.52
		FT8	2	15	8	16	13.00	6.50E+04	4.81	0.45
		FT9	2	13	15	8	12.00	6.00E+04	4.78	0.42
		FT10	2	15	11	10	12.00	6.00E+04	4.78	0.42
	Aviguard	Avi1	2	16	14	13	14.33	7.17E+04	4.86	0.50
		Avi2	2	21	16	18	18.33	9.17E+04	4.96	0.64
		Avi3	2	19	19	19	19.00	9.50E+04	4.98	0.66
		Avi4	2	7	11	12	10.00	5.00E+04	4.70	0.35
		Avi5	2	12	10	10	10.67	5.33E+04	4.73	0.37
		Avi6	2	21	15	19	18.33	9.17E+04	4.96	0.64
		Avi7	2	14	16	10	13.33	6.67E+04	4.82	0.47
		Avi8	2	11	15	15	13.67	6.83E+04	4.83	0.48
		Avi9	2	15	6	12	11.00	5.50E+04	4.74	0.38
		Avi10	2	18	25	14	19.00	9.50E+04	4.98	0.66
2	Inoculate count	N/A	5	2	6	3	3.67	1.83E+07	7.26	N/A
	MHB (Control))	C1	4	1	2	2	1.67	8.33E+05	5.92	4.55
		C2	3	15	13	10	12.67	6.33E+05	5.80	3.45
		C3	3	13	12	10	11.67	5.83E+05	5.77	3.18
		C4	3	5	5	9	6.33	3.17E+05	5.50	1.73
		C5	3	10	14	11	11.67	5.83E+05	5.77	3.18
		C6	3	14	10	11	11.67	5.83E+05	5.77	3.18
	CMT	CMT1	3	12	10	11	11.00	5.50E+05	5.74	3.00
		CMT2	3	12	6	10	9.33	4.67E+05	5.67	2.55
		CMT3	3	4	6	8	6.00	3.00E+05	5.48	1.64
		CMT4	3	9	10	10	9.67	4.83E+05	5.68	2.64
		CMT5	3	2	1	1	1.33	6.67E+04	4.82	0.36
		CMT6	3	10	4	10	8.00	4.00E+05	5.60	2.18
	Aviguard	Avi1	4	4	5	4	4.33	2.17E+06	6.34	11.82
		Avi2	4	2	2	2	2.00	1.00E+06	6.00	5.45
		Avi3	3	5	5	9	6.33	3.17E+05	5.50	1.73
		Avi4	4	2	2	2	2.00	1.00E+06	6.00	5.45
		Avi5	3	3	2	3	2.67	1.33E+05	5.12	0.73
		Avi6	3	9	8	5	7.33	3.67E+05	5.56	2.00
3	Inoculate count	N/A	4	13	7	3	7.67	3.83E+06	6.58	N/A
	MHB (Control)	C1	3	6	10	4	6.67	3.33E+05	5.52	8.70
		C2	3	7	4	3	4.67	2.33E+05	5.37	6.09
		C3	3	9	5	6	6.67	3.33E+05	5.52	8.70
		C4	3	7	3	4	4.67	2.33E+05	5.37	6.09
		C5	3	5	7	6	6.00	3.00E+05	5.48	7.83
		C6	3	7	5	4	5.33	2.67E+05	5.43	6.96
		C7	3	3	3	2	2.67	1.33E+05	5.12	3.48
		C8	3	4	3	2	3.00	1.50E+05	5.18	3.91
		C9	3	2	1	7	3.33	1.67E+05	5.22	4.35

		C10	4	4	3	4	3.67	1.83E+06	6.26	47.83
	CMT	FT1	3	3	3	6	4.00	2.00E+05	5.30	5.22
		FT2	3	3	4	3	3.33	1.67E+05	5.22	4.35
		FT3	3	5	4	7	5.33	2.67E+05	5.43	6.96
		FT4	2	15	16	16	15.67	7.83E+04	4.89	2.04
		FT5	3	6	6	4	5.33	2.67E+05	5.43	6.96
		FT6	3	1	3	3	2.33	1.17E+05	5.07	3.04
		FT7	2	6	5	8	6.33	3.17E+04	4.50	0.83
		FT8	2	8	6	8	7.33	3.67E+04	4.56	0.96
		FT9	2	10	12	9	10.33	5.17E+04	4.71	1.35
		FT10	2	13	12	14	13.00	6.50E+04	4.81	1.70
	Aviguard	Avi1	3	14	14	14	14.00	7.00E+05	5.85	18.26
		Avi2	3	5	8	7	6.67	3.33E+05	5.52	8.70
		Avi3	3	4	1	1	2.00	1.00E+05	5.00	2.61
		Avi4	2	17	23	19	19.67	9.83E+04	4.99	2.57
		Avi5	3	5	4	4	4.33	2.17E+05	5.34	5.65
		Avi6	3	6	5	7	6.00	3.00E+05	5.48	7.83
		Avi7	3	4	3	4	3.67	1.83E+05	5.26	4.78
		Avi8	3	6	2	2	3.33	1.67E+05	5.22	4.35
		Avi9	3	3	2	4	3.00	1.50E+05	5.18	3.91
		Avi10	3	2	2	2	2.00	1.00E+05	5.00	2.61

Table 49. Recovered *C. jejuni* 13126 and percentage invasion following GPA protocols on 8E11 cell lines

Invasion Repeat	Treatment	Replicate	Miles Misra dilution	Count 1	Count 2	Count 3	Average colony count	CFU/mL	Log10 CFU/mL	Invasion (%)
1	Inoculate Count	N/A	4	10	11	12	11.00	5.50E+06	6.74	N/A
	MHB (Control)	C1	3	13	7	13	11.00	5.50E+05	5.74	10.00
		C2	3	2	2	3	2.33	1.17E+05	5.07	2.12
		C3	3	6	5	2	4.33	2.17E+05	5.34	3.94
		C4	3	19	21	25	21.67	1.08E+06	6.03	19.70
		C5	3	9	9	10	9.33	4.67E+05	5.67	8.48
		C6	3	4	3	6	4.33	2.17E+05	5.34	3.94
		C7	3	4	5	3	4.00	2.00E+05	5.30	3.64
		C8	3	4	2	2	2.67	1.33E+05	5.12	2.42
		C9	3	5	2	1	2.67	1.33E+05	5.12	2.42
		C10	3	19	17	14	16.67	8.33E+05	5.92	15.15
	CMT	CMT1	3	3	5	5	4.33	2.17E+05	5.34	3.94
		CMT2	3	6	5	0	3.67	1.83E+05	5.26	3.33
		CMT3	3	3	3	2	2.67	1.33E+05	5.12	2.42
		CMT4	3	1	5	3	3.00	1.50E+05	5.18	2.73
		CMT5	3	5	7	1	4.33	2.17E+05	5.34	3.94
		CMT6	3	4	7	6	5.67	2.83E+05	5.45	5.15
		CMT7	3	4	4	4	4.00	2.00E+05	5.30	3.64
		CMT8	3	4	5	6	5.00	2.50E+05	5.40	4.55
		CMT9	3	4	2	5	3.67	1.83E+05	5.26	3.33
		CMT10	3	5	3	7	5.00	2.50E+05	5.40	4.55
	Aviguard	Avi1	3	5	7	2	4.67	2.33E+05	5.37	4.24
		Avi2	3	1	4	5	3.33	1.67E+05	5.22	3.03
		Avi3	3	5	5	6	5.33	2.67E+05	5.43	4.85
		Avi4	3	3	1	6	3.33	1.67E+05	5.22	3.03
		Avi5	3	7	18	17	14.00	7.00E+05	5.85	12.73
		Avi6	3	5	5	3	4.33	2.17E+05	5.34	3.94
		Avi7	3	8	4	21	11.00	5.50E+05	5.74	10.00
		Avi8	3	2	8	3	4.33	2.17E+05	5.34	3.94
		Avi9	3	2	2	5	3.00	1.50E+05	5.18	2.73
2	Inoculate Count	N/A	4	4	9	4	5.67	2.83E+06	6.45	N/A
	MHB (Control)	C1	3	2	2	1	1.67	8.33E+04	4.92	2.94
		C2	2	10	10	12	10.67	5.33E+04	4.73	1.88
		C3	2	9	13	8	10.00	5.00E+04	4.70	1.76
		C4	3	5	8	3	5.33	2.67E+05	5.43	9.41
		C5	3	1	4	1	2.00	1.00E+05	5.00	3.53
		C6	3	4	3	2	3.00	1.50E+05	5.18	5.29
		C7	3	1	2	1	1.33	6.67E+04	4.82	2.35
		C8	3	2	4	2	2.67	1.33E+05	5.12	4.71
		C9	3	2	1	1	1.33	6.67E+04	4.82	2.35
		C10	3	2	1	1	1.33	6.67E+04	4.82	2.35
	CMT	CMT1	2	13	18	9	13.33	6.67E+04	4.82	2.35
		CMT2	2	13	11	13	12.33	6.17E+04	4.79	2.18
		CMT3	2	6	11	8	8.33	4.17E+04	4.62	1.47
		CMT4	2	5	9	3	5.67	2.83E+04	4.45	1.00
		CMT5	2	6	9	9	8.00	4.00E+04	4.60	1.41
		CMT6	2	4	5	7	5.33	2.67E+04	4.43	0.94
		CMT7	3	1	4	2	2.33	1.17E+05	5.07	4.12
		CMT8	3	1	1	1	1.00	5.00E+04	4.70	1.76
		CMT9	3	3	2	3	2.67	1.33E+05	5.12	4.71
		CMT10	2	12	14	19	15.00	7.50E+04	4.88	2.65
	Aviguard	Avi1	3	4	3	2	3.00	1.50E+05	5.18	5.29
		Avi2	3	6	3	4	4.33	2.17E+05	5.34	7.65
		Avi3	2	11	16	15	14.00	7.00E+04	4.85	2.47
		Avi4	3	3	1	1	1.67	8.33E+04	4.92	2.94
		Avi5	3	2	2	2	2.00	1.00E+05	5.00	3.53
		Avi6	2	12	17	24	17.67	8.83E+04	4.95	3.12
		Avi7	3	1	2	1	1.33	6.67E+04	4.82	2.35
		Avi8	3	3	2	1	2.00	1.00E+05	5.00	3.53
		Avi9	3	5	2	5	4.00	2.00E+05	5.30	7.06
		Avi10	3	3	2	2	2.33	1.17E+05	5.07	4.12

Table 50. Recovered *S. Typhimurium* 4/74 and % invasion for GPA protocols on 8E11 cell lines

Infection strain	Treatment	Replicate	Miles Misra dilution	Count 1	Count 2	Count 3	Average colony count	CFU/mL	Log_{10} CFU/mL	Invasion (%)
1	Inoculate Count	N/A	6	5	10	8	7.67	3.83E+08	8.58	N/A
	LB (Control)	C1	5	16	18	8	14.00	7.00E+07	7.85	18.26
		C2	5	7	14	11	10.67	5.33E+07	7.73	13.91
		C3	5	7	5	4	5.33	2.67E+07	7.43	6.96
		C4	5	9	10	7	8.67	4.33E+07	7.64	11.30
		C5	5	7	8	6	7.00	3.50E+07	7.54	9.13
		C6	5	11	17	13	13.67	6.83E+07	7.83	17.83
		C7	5	14	16	19	16.33	8.17E+07	7.91	21.30
		C8	5	15	12	14	13.67	6.83E+07	7.83	17.83
	CMT	FT1	5	9	10	3	7.33	3.67E+07	7.56	9.57
		FT2	5	10	9	6	8.33	4.17E+07	7.62	10.87
		FT3	5	7	9	3	6.33	3.17E+07	7.50	8.26
		FT4	5	1	1	0	0.67	3.33E+06	6.52	0.87
		FT5	5	7	4	11	7.33	3.67E+07	7.56	9.57
		FT6	5	4	4	6	4.67	2.33E+07	7.37	6.09
		FT7	5	4	6	8	6.00	3.00E+07	7.48	7.83
		FT8	5	13	14	14	13.67	6.83E+07	7.83	17.83
		FT9	5	1	1	0	0.67	3.33E+06	6.52	0.87
		FT10	5	3	4	3	3.33	1.67E+07	7.22	4.35
	Aviguard	Avi1	5	9	9	11	9.67	4.83E+07	7.68	12.61
		Avi2	5	11	7	6	8.00	4.00E+07	7.60	10.43
		Avi3	5	10	5	4	6.33	3.17E+07	7.50	8.26
		Avi4	5	4	2	2	2.67	1.33E+07	7.12	3.48
		Avi5	5	5	4	1	3.33	1.67E+07	7.22	4.35
		Avi6	5	14	9	8	10.33	5.17E+07	7.71	13.48
		Avi7	5	4	4	2	3.33	1.67E+07	7.22	4.35
		Avi8	5	6	8	3	5.67	2.83E+07	7.45	7.39
		Avi9	5	2	5	6	4.33	2.17E+07	7.34	5.65
		Avi10	5	17	14	7	12.67	6.33E+07	7.80	16.52
2	Inoculate Count	N/A	6	17	23	16	18.67	9.33E+08	8.97	N/A
	LB (Control)	C1	6	1	2	1	1.33	6.67E+07	7.82	7.14
		C2	6	1	3	3	2.33	1.17E+08	8.07	12.50
		C3	6	1	1	3	1.67	8.33E+07	7.92	8.93
		C4	5	10	10	5	8.33	4.17E+07	7.62	4.46
		C5	6	1	1	3	1.67	8.33E+07	7.92	8.93
		C6	6	2	5	2	3.00	1.50E+08	8.18	16.07
		C7	5	6	5	5	5.33	2.67E+07	7.43	2.86
		C8	5	4	4	2	3.33	1.67E+07	7.22	1.79
		C9	5	6	11	4	7.00	3.50E+07	7.54	3.75
		C10	5	7	10	10	9.00	4.50E+07	7.65	4.82
	CMT	FT1	5	9	1	11	7.00	3.50E+07	7.54	3.75
		FT2	5	4	4	7	5.00	2.50E+07	7.40	2.68
		FT3	5	6	3	7	5.33	2.67E+07	7.43	2.86
		FT4	5	5	4	4	4.33	2.17E+07	7.34	2.32
		FT5	5	4	3	7	4.67	2.33E+07	7.37	2.50
		FT6	5	5	5	6	5.33	2.67E+07	7.43	2.86
		FT7	5	13	11	6	10.00	5.00E+07	7.70	5.36
		FT8	5	6	4	7	5.67	2.83E+07	7.45	3.04
		FT9	5	4	1	5	3.33	1.67E+07	7.22	1.79
		FT10	5	1	3	3	2.33	1.17E+07	7.07	1.25
	Aviguard	Avi1	5	7	9	10	8.67	4.33E+07	7.64	4.64
		Avi2	5	6	5	6	5.67	2.83E+07	7.45	3.04
		Avi3	5	5	8	5	6.00	3.00E+07	7.48	3.21
		Avi4	5	2	4	9	5.00	2.50E+07	7.40	2.68
		Avi5	5	11	9	11	10.33	5.17E+07	7.71	5.54
		Avi6	5	2	4	8	4.67	2.33E+07	7.37	2.50
		Avi7	5	2	9	5	5.33	2.67E+07	7.43	2.86
		Avi8	5	12	8	6	8.67	4.33E+07	7.64	4.64
		Avi9	5	3	8	8	6.33	3.17E+07	7.50	3.39
		Avi10	5	5	8	4	5.67	2.83E+07	7.45	3.04

Appendix 4: Appendix to Chapter 5

QIIME2™ commands for microbiota analysis

#input manifest file into QIIME2™ software
qiime tools import --type 'SampleData[PairedEndSequencesWithQuality]' --input-path manifest.csv --output-path Qiime2/paired-end-demux.qza --source-format PairedEndFastqManifestPhred33

 # Run DADA2 plugin
qiime dada2 denoise-paired –i--demultiplexed-seqs paired-end-demux.qza --p-trunc-len-f 250 --p-trunc-len-r 250 --p-trim-left-f 7 --p-trim-left-r 21 --output-dir DADA2

 # Visualise summary feature data
qiime feature-table summarize --i-table DADA2/table.qza --o-visualization table.qzv --m-sample-metadata-file mappingfile.tsv
qiime feature-table tabulate-seqs --i-data DADA2/representative_sequences.qza --o-visualization rep-seqs.qzv
qiime metadata tabulate --m-input-file DADA2/denoising_stats.qza --o-visualization denoising-stats.qzv

 # Run mafft pipeline
qiime phylogeny align-to-tree-mafft-fasttree –i- sequences DADA2/representative_sequences.qza –o-alignment aligned-rep-seqs.qza –o-masked-alignment masked-aligned-rep-seqs.qza –o- tree unrooted-tree.qza –o-rooted-tree rooted-tree.qza

#Alpha and Beta diversity analysis
qiime diversity core-metrics-phylogenetic --i-phylogeny rooted-tree.qza --i-table DADA2/table.qza --p-sampling-depth 44841 --m-metadata-file mappingfile.tsv --output-dir core-metrics-results
qiime diversity alpha-group-significance –i-alpha-diversity core-metrics-results/evenness_vector.qza –m-metadata-file mappingfile.tsv –o-visualization core-metrics-results/evenness-group-significance.qzv
qiime diversity alpha-group-significance –i-alpha-diversity core-metrics-results/observed_otus_vector.qza –m-metadata-file mappingfile.tsv –o-visualization core-metrics-results/observed-otus-group-significance.qzv
qiime diversity alpha-group-significance –i-alpha-diversity core-metrics-results/shannon_vector.qza –m-metadata-file mappingfile.tsv –o-visualization core-metrics-results/shannon-group-significance.qzv
qiime diversity beta-group-significance –i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza –m-metadata-file mappingfile.tsv –m-metadata-column Group –o-visualization core-metrics-results/unweighted-unifrac-group-significance.qzv –p-pairwise
qiime diversity beta-group-significance –i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza –m-metadata-file mappingfile.tsv –m-metadata-column Group –o-visualization core-metrics-results/weighted-unifrac-group-significance.qzv –p-pairwise

#Taxonomic analysis
qiime feature-classifier classify-sklearn --i-classifier gg-13-8-99-515-806-nb-classifier.qza --i-reads DADA2/representative_sequences.qza --o-classification taxonomy.qza
qiime metadata tabulate --m-input-file taxonomy.qza --o-visualization taxonomy.qzv
qiime taxa barplot --i-table DADA2/table.qza --i-taxonomy taxonomy.qza --m-metadata-file mappingfile.tsv --o-visualization taxa-bar-plots.qzv

Figure 83. QIIME2 System command list

Table 51. DNA quantities of samples for Illumina MiSEQ protocols. All samples are quantified using QuBit analysis

Sample	Organism	Quantification method	Concentration (ng/µl)	Sample volume (µl)
CMT1	Bacteria	QuBit	16.2	20
CMT2	Bacteria	QuBit	49.7	20
CMT3	Bacteria	QuBit	18.3	20
CMT4	Bacteria	QuBit	14.3	20
CMT5	Bacteria	QuBit	19.9	20
CMT6	Bacteria	QuBit	12.3	20
CMT7	Bacteria	QuBit	8.66	20
CMT8	Bacteria	QuBit	8.77	20
CMT9	Bacteria	QuBit	6.66	20
CMT10	Bacteria	QuBit	5.25	20
Ext. Control 1	Bacteria	QuBit	24.9	20
Ext. Control 2	Bacteria	QuBit	14.7	20
Ext. Control 3	Bacteria	QuBit	7.34	20
Ext. Control 4	Bacteria	QuBit	9.63	20
Ext. Control 5	Bacteria	QuBit	10.7	20
Ext. Control 6	Bacteria	QuBit	27.8	20
Ext. Control 7	Bacteria	QuBit	7.77	20
Ext. Control 8	Bacteria	QuBit	4.32	20
Ext. Control 9	Bacteria	QuBit	8.06	20
Ext. Control 10	Bacteria	QuBit	6.9	20
Int. Control 1	Bacteria	QuBit	15.1	20
Int. Control 2	Bacteria	QuBit	16.1	20
Int. Control 3	Bacteria	QuBit	19.7	20
Int. Control 4	Bacteria	QuBit	22.2	20
Int. Control 5	Bacteria	QuBit	14.9	20
Int. Control 6	Bacteria	QuBit	11.8	20
Int. Control 7	Bacteria	QuBit	11.2	20
Int. Control 8	Bacteria	QuBit	16.1	20
Int. Control 9	Bacteria	QuBit	0.419	20
Int. Control 10	Bacteria	QuBit	9.68	20
Aviguard 1	Bacteria	QuBit	74.8	20
Aviguard 2	Bacteria	QuBit	7.92	20
Aviguard 3	Bacteria	QuBit	35	20
Aviguard 4	Bacteria	QuBit	22.2	20
Aviguard 5	Bacteria	QuBit	18.5	20
Aviguard 6	Bacteria	QuBit	29.6	20
Aviguard 7	Bacteria	QuBit	46.8	20
Aviguard 8	Bacteria	QuBit	49.2	20
Aviguard 9	Bacteria	QuBit	10.8	20
Aviguard 10	Bacteria	QuBit	17.4	20
3d_CMT1	Bacteria	QuBit	27.2	20
3d_CMT2	Bacteria	QuBit	14.9	20
3d_CMT3	Bacteria	QuBit	26.3	20
3d_CMT4	Bacteria	QuBit	35.8	20
3d_Aviguard1	Bacteria	QuBit	7.53	20
3d_Aviguard2	Bacteria	QuBit	33.8	20
3d_Aviguard3	Bacteria	QuBit	10.3	20
3d_Ext. Control 1	Bacteria	QuBit	32.7	20
3d_Ext. Control 2	Bacteria	QuBit	32.2	20
3d_Ext. Control 3	Bacteria	QuBit	20.7	20
3d_Int. Control 1	Bacteria	QuBit	23.6	20
3d_Int. Control 2	Bacteria	QuBit	2.39	20
3d_Int. Control 3	Bacteria	QuBit	27.1	20
AV_MAT_2	Bacteria	QuBit	3.21	20
CMT_MAT_1	Bacteria	QuBit	5.72	20
CMT_MAT_2	Bacteria	QuBit	5.88	20

Table 52. Number of sequences for each processed sample following Illumina MiSeq protocols.

Sample ID	Treatment Group	Age	Sequence Count
CMTMAT1	-	-	87207
CMTMAT2	-	-	64083
AvMAT 1	-	-	80457
CMT1	CMT treated	7 d.p.h	111568
CMT2	CMT treated	7 d.p.h	59022
CMT3	CMT treated	7 d.p.h	55625
CMT4	CMT treated	7 d.p.h	54917
CMT5	CMT treated	7 d.p.h	100721
CMT6	CMT treated	7 d.p.h	120917
CMT7	CMT treated	7 d.p.h	94670
CMT8	CMT treated	7 d.p.h	90632
CMT9	CMT treated	7 d.p.h	100074
CMT10	CMT treated	7 d.p.h	69917
H1	Ext. Control	7 d.p.h	88073
H2	Ext. Control	7 d.p.h	107988
H3	Ext. Control	7 d.p.h	115389
H4	Ext. Control	7 d.p.h	78548
H5	Ext. Control	7 d.p.h	68204
H6	Ext. Control	7 d.p.h	44841
H7	Ext. Control	7 d.p.h	105395
H8	Ext. Control	7 d.p.h	95182
H9	Ext. Control	7 d.p.h	102740
H10	Ext. Control	7 d.p.h	103356
C1	Int. Control	7 d.p.h	101033
C2	Int. Control	7 d.p.h	123989
C3	Int. Control	7 d.p.h	77929
C4	Int. Control	7 d.p.h	85493
C5	Int. Control	7 d.p.h	106937
C6	Int. Control	7 d.p.h	90290
C7	Int. Control	7 d.p.h	82793
C8	Int. Control	7 d.p.h	93334
C9	Int. Control	7 d.p.h	44963
C10	Int. Control	7 d.p.h	92565
A1	Aviguard® Treated	7 d.p.h	96183
A2	Aviguard® Treated	7 d.p.h	118325
A3	Aviguard® Treated	7 d.p.h	75630
A4	Aviguard® Treated	7 d.p.h	63201
A5	Aviguard® Treated	7 d.p.h	60388
A6	Aviguard® Treated	7 d.p.h	112800
A7	Aviguard® Treated	7 d.p.h	111525
A8	Aviguard® Treated	7 d.p.h	76378
A9	Aviguard® Treated	7 d.p.h	49008
A10	Aviguard® Treated	7 d.p.h	70109
3d_CMT1	CMT Treated	3 d.p.h	96394
3d_CMT2	CMT Treated	3 d.p.h	84915
3d_CMT3	CMT Treated	3 d.p.h	105610
3d_CMT4	CMT Treated	3 d.p.h	71829
3d_A1	Aviguard® Treated	3 d.p.h	84743
3d_A2	Aviguard® Treated	3 d.p.h	89610
3d_A3	Aviguard® Treated	3 d.p.h	89320
3d_H1	Ext. Control	3 d.p.h	91907
3d_H2	Ext. Control	3 d.p.h	115061
3d_H3	Ext. Control	3 d.p.h	79881
3d_C1	Int. Control	3 d.p.h	71315
3d_C2	Int. Control	3 d.p.h	66630
3d_C3	Int. Control	3 d.p.h	113937

Table 53. Alpha diversity statistics shown for observed out, Shannon and Pielou evenness metrics.

Sample ID	Treatment Group	Age (d.p.h)	Metric		
			observed_otus	Shannon	Pielou
CMT1	CMT	7	121	5.141849	0.743164
CMT2	CMT	7	118	5.243579	0.761855
CMT3	CMT	7	124	5.340499	0.767954
CMT4	CMT	7	100	4.60395	0.692964
CMT5	CMT	7	101	4.365535	0.655662
CMT6	CMT	7	204	5.775629	0.752777
CMT7	CMT	7	114	3.906736	0.571755
CMT8	CMT	7	175	4.492313	0.602897
CMT9	CMT	7	82	3.718769	0.584937
CMT10	CMT	7	190	5.329622	0.704059
Ext. Control 1	Ext. Control	7	31	2.646616	0.534217
Ext. Control 2	Ext. Control	7	40	3.066644	0.576228
Ext. Control 3	Ext. Control	7	28	2.941746	0.611926
Ext. Control 4	Ext. Control	7	38	2.703969	0.515245
Ext. Control 5	Ext. Control	7	33	2.575939	0.510654
Ext. Control 6	Ext. Control	7	36	3.184915	0.616047
Ext. Control 7	Ext. Control	7	33	2.792606	0.553606
Ext. Control 8	Ext. Control	7	35	2.33315	0.454869
Ext. Control 9	Ext. Control	7	42	2.97532	0.55177
Ext. Control 10	Ext. Control	7	40	3.032399	0.569793
Int. Control 1	Int. Control	7	42	2.570053	0.476614
Int. Control 2	Int. Control	7	23	2.583275	0.571071
Int. Control 3	Int. Control	7	76	2.365656	0.378631
Int. Control 4	Int. Control	7	16	1.620137	0.405034
Int. Control 5	Int. Control	7	18	1.93333	0.463637
Int. Control 6	Int. Control	7	21	2.538704	0.577987
Int. Control 7	Int. Control	7	22	2.710189	0.607743
Int. Control 8	Int. Control	7	18	2.432929	0.583447
Int. Control 9	Int. Control	7	32	2.742669	0.548534
Int. Control 10	Int. Control	7	26	2.873798	0.611389
Aviguard 1	Aviguard	7	54	3.857191	0.670246
Aviguard 2	Aviguard	7	48	2.141492	0.383439
Aviguard 3	Aviguard	7	56	4.19332	0.722071
Aviguard 4	Aviguard	7	43	3.110422	0.573216
Aviguard 5	Aviguard	7	60	4.143292	0.701434
Aviguard 6	Aviguard	7	49	3.625462	0.645708
Aviguard 7	Aviguard	7	52	3.408657	0.597964
Aviguard 8	Aviguard	7	63	4.560059	0.762899
Aviguard 9	Aviguard	7	61	4.558576	0.768636
Aviguard 10	Aviguard	7	51	3.949838	0.696323
3d_CMT1	CMT	3	58	3.196973	0.545747
3d_CMT2	CMT	3	29	2.427429	0.499679
3d_CMT3	CMT	3	114	4.3293	0.633597
3d_CMT4	CMT	3	65	3.953652	0.656495
3d_Aviguard1	Aviguard	3	39	3.27769	0.62014
3d_Aviguard2	Aviguard	3	36	3.598092	0.695966
3d_Aviguard3	Aviguard	3	43	3.553481	0.654867
3d_Ext. Control 1	Ext. Control	3	26	2.336354	0.49705
3d_Ext. Control 2	Ext. Control	3	23	1.612776	0.356528
3d_Ext. Control 3	Ext. Control	3	22	2.04975	0.459644
3d_Int. Control 1	Int. Control	3	15	2.203958	0.564121
3d_Int. Control 2	Int. Control	3	8	1.441641	0.480547
3d_Int. Control 3	Int. Control	3	19	2.344251	0.551858
AV_MAT_1	AvMat	N/A	92	4.665639	0.715198
CMT_MAT_1	CMTMat	N/A	362	6.458213	0.759804
CMT_MAT_2	CMTMat	N/A	343	6.529707	0.77531